

1 IAP5 Rec'd PCT/PTO 10 FEB 2006
10/567749**A Process for Preparing L-Threonine**Cross Reference to Related Applications

The present application represents U.S. national stage of international application PCT/EP2004/008470, which had an 5 international filing date of July 29, 2004, and which was published in English under PCT Article 21(2) on February 17, 2005. The international application claims priority to German applications 103 37 028.5, filed on August 13, 2003; and 10 2004 029 639.1, filed on June 18, 2004. The international 10 application also claims priority to United States provisional application 60/494,566, filed on August 13, 2003. These prior applications are hereby incorporated by reference in their entirety.

Field of the Invention

15 The invention provides an improved process for the fermentative preparation of L-threonine using bacteria from the family Enterobacteriaceae.

Background of the Invention

L-threonine is used in human medicine, in the pharmaceutical industry, in the foodstuffs industry and very particularly in animal nutrition. 20

It is known that L-threonine can be prepared by fermentation from strains of the family Enterobacteriaceae, in particular Escherichia coli. Due to the great importance of this amino acid, efforts are constantly made to improve the method of preparation. Process improvements may be based on 25 fermentation technology steps, such as e.g. stirring and supplying with oxygen, or the composition of the nutrient medium, such as e.g. the sugar concentration during 30 fermentation, or working up to give the final product by e.g. ion exchange chromatography or the intrinsic, i.e.

genetically based, performance characteristics of the bacterium itself.

US-A-5,538,873 and EP-B-0593792 or Okamoto et al.

(Bioscience, Biotechnology, and Biochemistry 61 (11),

5 1877 - 1882, 1997) describe how threonine can be prepared by fermentation in a batch process or a fed batch process.

Furthermore, US 6,562,601 describes a process for preparing L-threonine using strains of the family Enterobacteriaceae in which, after performing fermentation in a fed batch process,

10 the fermentation broth is drained down to 1-90 vol.%, then the remaining fermentation broth is topped up with growth medium and, preferably after a growth phase, a further fermentation step is performed by the fed batch process mentioned. This process may be repeated several times and is

15 therefore called a repeated fed batch process.

Another process for preparing threonine using bacteria from the family Enterobacteriaceae, in particular Escherichia coli, is described in the patent US 6,562,601. This comprises first cultivating the bacterium in a fed batch process, 20 wherein threonine is enriched in the fermentation broth. At a desired time, some, i.e. 10 to 99% of the fermentation broth present in the fermenter, is harvested. The remainder of the fermentation broth remains in the fermenter. The fermentation broth remaining in the fermenter is topped up with nutrient medium and another fermentation is performed using the fed 25 batch process. The cycle described is optionally performed several times.

Object of the Invention

The object of the invention is to provide new measures for 30 the improved fermentative preparation of L-threonine.

Summary of the Invention

The invention provides a fermentation process, characterized in that

- a) the bacterium is inoculated into at least a first nutrient medium and cultivated, then
- b) some of the fermentation broth is abstracted, wherein more than 90 vol.%, in particular more than 91 vol.%,
5 more than 92 vol.%, more than 93 vol.%, more than 94 vol.%, more than 95 vol.%, more than 96 vol.%, more than 97 vol.% or more than 98 vol.% of the total volume of fermentation broth remains in the fermentation container and wherein a maximum of 99 vol.%, 99.3 vol.%, 99.6 vol.%
10 or 99.9 vol.% of the total volume of the fermentation broth remains in the fermentation container, then
- c) the remaining fermentation broth is topped up with one or more further nutrient media, wherein the further nutrient medium or further nutrient media contains at least one source of carbon, at least one source of nitrogen and at least one source of phosphorus, and cultivation is continued under conditions which enable the formation of L-threonine,
15
- d) steps b) and c) are optionally performed several times,
20 and
- e) the concentration of the source(s) of carbon during cultivation in accordance with step c) and/or d) is adjusted to a maximum of 30 g/l.

Detailed Description of the Invention

25 Cultivation of the bacterium in accordance with step a) is performed typically in a fermenter (bioreactor). These have a volume of about 10 - 500 m³ (cubic meters) on an industrial production scale. On a laboratory scale, when the process according to the invention can be checked in a simple manner,
30 fermenter volumes of 1 - 50 l are typical. Fermenter volumes of 50 l to 10 m³ are normally used on a pilot-plant scale.

The expression plant performance is understood to mean that the weight or amount of a product is produced with a certain yield and at a certain rate or with a certain productivity or space-time yield in a plant such as e.g. a fermenter. These 5 parameters largely determine the cost or economic viability of a process.

A fermentation broth is understood to be the suspension of a microorganism being produced by the cultivation of a microorganism, in the case of the present invention a 10 L-threonine-producing bacterium, in a nutrient medium using a fermenter.

According to the invention, the plant performance of a L-threonine-producing fermenter can be increased by cultivating by the batch process or the fed batch process in 15 the first step a) described above, wherein when using the fed batch process at least one additional nutrient medium is used. In step b) described above, the culture fermentation broth is withdrawn, wherein less than 10 vol.%, in particular less than 9 vol.%, less than 8 vol.%, less than 7 vol.%, less 20 than 6 vol.%, less than 5 vol.%, less than 4 vol.%, less than 3 vol.%, less than 2 vol.% of the total volume of the fermentation broth is abstracted, and wherein a minimum of 1 vol.%, 0.7 vol.%, 0.4 vol.% or 0.1 vol.% of the total volume of the fermentation broth is abstracted. Accordingly, more 25 than 90 up to a maximum of 99.9 vol.% of the fermentation broth remains in the fermenter in the process according to the invention, in accordance with step b).

Then, in step c) the remaining fermentation broth is topped up with one or more further nutrient media, up to about 100% 30 of the original volume, wherein the further nutrient medium or further nutrient media contains at least one source of carbon, at least one source of nitrogen and at least one source of phosphorus, and cultivation continues under conditions which enable the formation of L-threonine. This

step c) is optionally repeated several times. The L-threonine formed is collected and optionally purified and isolated.

During cultivation step a), the bacterium is inoculated into at least a first nutrient medium and is cultivated by the
5 batch process or the fed batch process. When using the fed batch process, an added nutrient medium is supplied after more than 0 up to a maximum of 10 hours, advantageously after 1 to 10 hours, preferably after 2 to 10 hours and particularly preferably after 3 to 7 hours.

10 The first nutrient medium contains, as a source of carbon, one or more compounds chosen from the group saccharose, molasses from sugar beet or sugar cane, fructose, glucose, starch hydrolysate, lactose, galactose, maltose, xylose, cellulose hydrolysate, arabinose, acetic acid, ethanol and
15 methanol in concentrations of 1 to 100 g/kg or 1 to 50 g/kg, preferably 10 to 45 g/kg, particularly preferably 20 to 40 g/kg. Starch hydrolysate is understood to mean the hydrolysate from corn, cereals, potatoes or tapioca.

Sources of nitrogen which can be used in the first nutrient
20 medium may be organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soy bean flour and urea or inorganic compounds such as ammonia, ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate,
25 potassium nitrate, potassium sodium nitrate. The sources of nitrogen may be used individually or as a mixture in concentrations of 1 to 40 g/kg, preferably 1 to 30 g/kg or 10 to 30 g/kg, particularly preferably 1 to 25 g/kg or 10 to 25 g/kg, very particularly preferably 1 to 30 g/kg or 1 to 25
30 g/kg.

Sources of phosphorus which may be used in the first nutrient medium are phosphoric acid, alkali metal or alkaline earth metal salts of phosphoric acid, in particular potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the

corresponding sodium-containing salts, polymers of phosphoric acid or the hexaphosphate of inositol, also called phytic acid, or the alkali metal or alkaline earth metal salts thereof in concentrations of 0.1 to 5 g/kg, preferably 0.3 to 5 3 g/kg, particularly preferably 0.5 to 1.5 g/kg. The first nutrient medium must also contain salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are required for growth. These substances are present in concentrations of 0.003 to 3 g/kg. Finally, essential growth substances such as 10 amino acids (e.g. homoserine) and vitamins (e.g. thiamine) are used in addition to the substances mentioned above. Antifoaming agents, such as e.g. polyglycol esters of fatty acids, may also be used to control the production of foam.

The added nutrient medium which is used in a fed batch 15 process generally contains, simply as a source of carbon, one or more of the compounds chosen from the group saccharose, molasses from sugar beet or sugar cane, fructose, glucose, starch hydrolysate, lactose, galactose, maltose, xylose, cellulose hydrolysate, arabinose, acetic acid, ethanol and 20 methanol in concentrations of 300 to 700 g/kg, preferably 400 to 650 g/kg, and optionally an inorganic source of nitrogen such as e.g. ammonia, ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate, ammonium nitrate, potassium nitrate or potassium sodium nitrate. Alternatively, 25 these and other components may also be fed separately.

It was found that in the process according to the invention, in accordance with step c) and/or d), the constituents of the further nutrient medium may be supplied to the culture in the form of a single further nutrient medium as well as in a 30 number of further nutrient media. According to the invention, the further nutrient medium is or the further nutrient media are supplied to the culture in at least one (1) feed stream or in a number of feed streams in least 2 to 10, preferably 2 to 7 or 2 to 5 feed streams.

The further nutrient medium or the further nutrient media contain(s), as a source of carbon, one or more compounds chosen from the group saccharose, molasses from sugar beet or sugar cane, fructose, glucose, starch hydrolysate, maltose,
5 xylose, cellulose hydrolysate, arabinose, acetic acid, ethanol and methanol in concentrations of 20 to 700 g/kg, preferably 50 to 650 g/kg.

Furthermore, the further nutrient medium contains or the further nutrient media contain a source of nitrogen
10 consisting of organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soy bean flour and urea or inorganic compounds such as ammonia, ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate, ammonium nitrate
15 and/or potassium nitrate or potassium sodium nitrate. The sources of nitrogen may be used individually or as a mixture in concentrations of 5 to 50 g/kg, preferably 10 to 40 g/kg.

Furthermore, the further nutrient medium contains or the further nutrient media contain a source of phosphorus
20 consisting of phosphoric acid or the alkali metal or alkaline earth metal salts of phosphoric acid, in particular potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts, polymers of phosphoric acid or the hexaphosphate of inositol, also known as phytic acid, or the corresponding alkali metal or alkaline earth metal salts. The sources of phosphorus may be used individually or as a mixture in concentrations of 0.3 to 3 g/kg, preferably 0.5 to 2 g/kg. The further nutrient medium or further nutrient media must also contain salts of metals,
25 such as e.g. magnesium sulfate or iron sulfate, which are required for growth, in concentrations of 0.003 to 3 g/kg, preferably in concentrations of 0.008 to 2 g/kg. Finally, essential growth substances such as amino acids (e.g. homoserine) and vitamins (e.g. thiamine) are used in addition
30 to the substances mentioned above. Antifoaming agents, such
35 to the substances mentioned above. Antifoaming agents, such

as e.g. polyglycol esters of fatty acids, may also used to control the production of foam.

When using a single further nutrient medium, this is typically supplied to the culture in one feed stream. When 5 using a number of further nutrient media, these are supplied in a corresponding number of feed streams. When using a number of further nutrient media, it should be noted that each of these may contain only one of the sources of carbon, nitrogen or phosphorus mentioned, or else a mixture of the 10 sources of carbon, nitrogen or phosphorus mentioned.

According to the invention, the fed further nutrient medium or the fed further nutrient media is adjusted in such a way that a phosphorus to carbon ratio (P/C ratio) of at most 4; of at most 3; of at most 2; of at most 1.5; of at most 1; of 15 at most 0.7; of at most 0.5; at most 0.48; at most 0.46; at most 0.44; at most 0.42; at most 0.40; at most 0.38; at most 0.36; at most 0.34; at most 0.32; at most 0.30 mmoles of phosphorus per mole of carbon is present.

The abstraction of fermentation broth described in step b) 20 takes place in less than 180 minutes, preferably in less than 120 minutes, particularly preferably in less than 60 minutes and very particularly preferably in less than 30 to less than 15 minutes.

If a further nutrient medium or several further nutrient 25 media are used for topping up as described in step c), this topping up may take place in the form of one or several batches or feedstocks or continuously or using a combination of the two procedures. A final top-up level of about 100% of the original volume is again reached. The expression "about 30 100%" in this context means that some variations may occur within the scope of the technical possibilities which may lead to the final top-up level being, for example, 97%-103%, 98%-102%, 99-101%, 99.5-100.5% or 99.9-100.1% of the original volume.

If topping up takes place in the form of one or several batches, this occurs, according to the invention, as rapidly as possible i.e. in less than 180 minutes, preferably in less than 120 minutes, particularly preferably in less than 60 minutes, particularly preferably in less than 30 minutes to less than 15 minutes. After topping-up to about 100% of the original volume as described above, cultivation takes place until the source of carbon has been consumed or up to another suitable time shortly before complete consumption of the source of carbon, before again abstracting fermentation broth in accordance with step b). At this point, the concentration of the source of carbon is > 0 to ≤ 5 g/l, > 0 to ≤ 3 g/l, > 0 to ≤ 2 g/l, > 0 to ≤ 1 g/l, > 0 to ≤ 0.5 g/l.

During a continuous topping up procedure, then topping up with one or more further nutrients takes place until approximately 100% of the original volume is reached again. The fermentation broth is then cultivated further until the source of carbon has been consumed or almost (see above) consumed.

When using a combination of the two procedures one or more further nutrient media in the form of one or more batches are added as rapidly as possible and then one or more further nutrient media are introduced continuously with continuing cultivation. The fermentation broth is cultivated further until the source of carbon has been consumed or almost (see above) consumed.

Cultivation in steps a) and c) is performed under conditions which enable the formation of L-threonine. During cultivation the temperature is adjusted to be within the range 27 to 45°C, preferably 29 to 42°C, particularly preferably 33 to 40°C. Fermentation can be performed at atmospheric pressure or optionally under an excess pressure, preferably at 0 to 2.5 bar excess pressure, particularly preferably at 0 to 1.5 bar. The oxygen partial pressure is regulated to 5 to 50%, preferably about 20%, of the saturation value for air.

Controlling the pH to a value of about 6 to 8, preferably 6.5 to 7.5 can be performed with 25% strength ammonia water. The conditions for cultivation may remain constant or may alter during cultivation. Likewise, the cultivation conditions in 5 steps a) and c) may be identical or different.

Repeating steps b) and c) in accordance with d) takes place > (greater than) 0 to 100 times, preferably 2 to 90 or 2 to 80 times, particularly preferably 4 to 70, 4 to 60, 4 to 50 or 4 to 40 times and particularly preferably 5 to 30, 6 to 30, 7 10 to 30, 8 to 30, 9 to 30 or 10 to 30 times.

The time between abstracting at least 0.1 vol.% to less than 10 vol.% of the total volume of fermentation broth, complete topping up to about 100%, subsequent cultivation and renewed abstraction of the fermentation broth is at most 10 hours or 15 at most 5 hours, preferably at most 3 hours, particularly preferably at most 2 hours to at most 1 hour.

Accordingly, abstraction of the fermentation broth, topping up with nutrient medium, subsequent cultivation and renewed abstraction of fermentation broth takes place at a rate which 20 corresponds to an average residence time of less than 100 hours or less than 50 hours, preferably less than 30, very particularly preferably less than 20 or less than 10 hours. The average residence time is the theoretical time that the particles remain within a culture. The average residence time 25 is described by the ratio of the volume of liquid in the reactor to the amount which flows through (Biotechnologie; H. Weide, J. Páca and W. A. Knorre; Gustav Fischer Verlag Jena; 1991). The amount which flows through is defined by the volume of fermentation broth drained off or the volume of 30 nutrient medium or further nutrient media used for topping up. Measurement of the full status can be performed directly, e.g. using a radar measurement, or indirectly, e.g. using a weight determination.

According to the invention, the concentration of the source of carbon during cultivation in accordance with step c) and/or d) is adjusted in general to at most 30 g/l, to at most 20 g/l, to at most 10 g/l, preferably to at most 5 g/l,
5 particularly preferably at most 2 g/l. This concentration is held steady for at least 75%, preferably for at least 85%, particularly preferably for at least 95% of the time of cultivation in accordance with step b) and/or c). The concentration of the source of carbon is determined using
10 methods which are disclosed in the prior art. β -D-glucose is determined, for example, in a glucose analyzer, YSI 02700 Select, from Yellow Springs Instruments (Yellow Springs, Ohio, USA).

Optionally, the withdrawn culture broth can be provided with
15 oxygen or an oxygen-containing gas, optionally with stirring, until the concentration of the source of carbon falls to below 2 g/l; below 1 g/l; or below 0.5 g/l.

In a process according to the invention, the yield is at least 31%; at least 33%; at least 35%; at least 37%; at least
20 40%, at least 42%; at least 44%; at least 46%; at least 48%. Here, the yield is defined as the ratio of the total amount of L-threonine formed in a cultivation process to the total amount of the source of carbon used or consumed.

In a process according to the invention, L-threonine is formed with a space-time yield of at least 1.5 to 2.5 g/l per hr., at least 2.5 to 3.5 g/l per hr., at least 2.5 to more than 3.5 g/l per hr., at least 3.5 to 5.0 g/l per hr., at least 3.5 to more than 5.0 g/l per hr., or at least 5.0 to 8.0 g/l or more per hr. The space-time yield is defined as
30 the ratio of the total amount of threonine formed in a cultivation process to the volume of the culture, regarded over the entire time of cultivation. The space-time yield is also known as the volumetric productivity.

Naturally, in a fermentation process like the one according to the invention, the product is produced with a certain yield and with a certain space-time yield (volumetric productivity). In a process according to the invention, L-threonine can be produced with a yield of at least 31% and a space-time yield of at least 1.5 to 2.0 g/l per hour. Further couplings of yield and space-time yield such as for example a yield of at least 37% and a space-time yield of at least 2.5 g/l per hour are easily produced from the specifications given above.

L-threonine can be recovered, collected or concentrated from the withdrawn culture broth and optionally purified.

It is also possible to produce a product from the withdrawn culture broth (= fermentation broth) by removing the biomass of bacterium present in the culture broth completely (100%) or almost completely i.e. by removing more than or greater than (>) 90%, 95%, 97%, 99% of the biomass and largely leaving behind the other constituents of the fermentation broth, i.e. leaving 30%-100%, 40%-100%, 50%-100%, 60%-100%, 70%-100%, 80%-100% or 90%-100% of these, preferably greater than or equal to (\geq) 50%, \geq 60%, \geq 70%, \geq 80%, \geq 90% or \geq 95% of these or even the entire amount (100%) of these in the product.

Separation methods such as for example centrifuging, filtering, decanting, flocculating or a combination of these are used to remove or isolate the biomass.

The broth obtained is then thickened or concentrated using known methods such as for example by using a rotary evaporator, thin layer evaporator or falling film evaporator, by reverse osmosis, by nanofiltration or by a combination of these.

This concentrated broth is then processed using the methods of freeze-drying, spray-drying, spray granulation or any other process to give a preferably free flowing, finely

divided powder. This free-flowing finely divided powder can then again be converted into a coarse-grained, very free-flowing, storable and largely dust-free product by using suitable compacting or granulating processes. Altogether,
5 more than 90% of the water is removed in this way so that the water content of the product is less than 10%, less than 5%.

The process steps mentioned above do not necessarily have to be performed in the sequence specified here, but they may optionally be combined in a technically meaningful manner.

10 The process according to the invention is characterized in particular by an increased space-time yield when compared with a conventional fed batch process.

Analysis of L-threonine and other amino acids may be performed by anion exchange chromatography followed by
15 ninhydrin derivation as described in Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958)) or by reversed phase HPLC as described in Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

To perform the process according to the invention, L-
20 threonine-producing bacteria from the family Enterobacteriaceae, chosen from the genera Escherichia, Erwinia, Providencia and Serratia are suitable. The genera Escherichia and Serratia are preferred. From the genus Escherichia the species Escherichia coli is mentioned in
25 particular and from the genus Serratia the species Serratia marcescens is mentioned in particular.

The bacteria contain at least one copy of a thrA gene or allele which codes for a threonine-insensitive aspartate kinase I - homoserine dehydrogenase I. In this connection,
30 the literature mentions "feed back" resistant or even desensitized variants. These types of bacteria are typically resistant to the threonine analog α-amino-β-hydroxyvaleric acid (AHV) (Shio and Nakamori, Agricultural and Biological Chemistry 33 (8), 1152-1160 (1969)). Biochemical tests

relating to "feed back" resistant aspartate kinase I - homoserine dehydrogenase I variants are described for example in Cohen et al. (Biochemical and Biophysical Research Communications 19(4), 546-550 (1965)) and in Omori et al.

5 (Journal of Bacteriology 175(3), 785-794 (1993)). Optionally, the threonine-insensitive aspartate kinase I - homoserine dehydrogenase I is overexpressed.

Methods of overexpression are adequately described in the prior art, for example in Makrides et al. (Microbiological

10 Reviews 60 (3), 512-538 (1996)). The copy number is raised by at least one (1) copy by using vectors. Plasmids such as for example those described in US 5,538,873 can be used as vectors. Phages, for example the phage Mu, as described in EP 0 332 448, or the phage lambda (λ) can also be used as 15 vectors. An increase in the copy number can also be produced by incorporating a further copy at another site on the chromosome, for example at the att site on the phage λ (Yu and Court, Gene 223, 77-81 (1998)). US 5,939,307 describes how an increase in expression can be produced by

20 incorporating expression cassettes or promoters such as for example the tac promoter, trp promoter, lpp promoter or P_L promoter and P_R promoter upstream of the phage λ in the chromosomal threonine operon. Promoters in the phage T7, gear-box promoters or the nar promoter can also be used in 25 the same way. These types of expression cassettes or promoters can also be used by overexpressing plasmid-bonded genes, as described in EP 0 593 792. There again, the expression of plasmid-bonded genes can be regulated by using the lacI^Q allele (Glascock and Weickert, Gene 223, 221-231

30 (1998)). Overexpression can also be produced by removing the attenuator in the threonine operon (Park et al., Biotechnology Letters 24, 1815-1819 (2002)) or by using the thr79-20 mutation (Gardner, Proceedings of the National Academy of Sciences, USA 76(4), 1706-1710 (1979)) or by 35 mutation of the thrS gene coding for threonyl-t-RNA synthetase as described in Johnson et al. (Journal of

Bacteriology 129(1), 66-70 (1977)). Using the measures described, the intracellular concentration of the particular aspartate kinase I - homoserine dehydrogenase I protein variants is increased by at least 10% as compared with the 5 starting strain.

A suitable *thrA* allele is described in US 4,278,765 and is obtainable in the form of the strain MG442 from the Russian National Collection of Industrial Microorganisms (VKPM, Moscow, Russia) under accession number CMIM B-1628. Other 10 suitable *thrA* alleles are described in WO 00/09660 and WO 00/09661 and are obtainable from the Korean Culture Centre for Microorganisms (KCCM, Seoul, Korea) under accession numbers KCCM 10132 and KCCM 10133. Another suitable *thrA* allele is present in the strain H-4581, which is described in 15 US 4,996,147 and is obtainable under accession number Ferm BP-1411 from the National Institute of Advanced Industrial Science and Technology (1-1-1 Higashi, Tsukuba Ibaraki, Japan). Finally, further *thrA* alleles are described in US 3,580,810 and these are obtainable in the form of strains 20 ATCC 21277 and ATCC 21278 deposited at ATCC. Another allele is described in US 3,622,453 and is obtainable from ATCC in the form of strain KY8284, under accession number ATCC 21272. In addition, another *thrA* allele is described in WO 02/064808 and is deposited at KCCM in the form of strain pGmTN-PPC12, 25 under accession number KCCM 10236.

Optionally, *thrA* alleles which code for "feed back" resistant aspartate kinase I - homoserine dehydrogenase I variants can be isolated using the adequately well-known methods of mutagenesis of cells using mutagenic substances, for example 30 N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) or ethylmethane sulfonate (EMS) or mutagenic radiation, for example UV radiation followed by selection of threonine analog (for example AHV) resistant variants. These types of mutagenesis methods are described, for example, in Shio and Nakamori 35 (Agricultural and Biological Chemistry 33 (8), 1152-1160 (1969)) or in Saint-Girons and Margerita (Molecular and

General Genetics 162, 101-107 (1978)) or in the well-known manual by J.H. Miller (A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria, Cold Spring Harbor Laboratory Press, New York, USA, 1992) in particular on pages 135 to 156. Shioi and Nakamori, for example, treat a cell suspension of Escherichia coli with 0.5 mg/ml of MNNG in a 0.1 M sodium phosphate buffer at pH 7 for about 15 minutes at room temperature (i.e. in general at about 16 to 26°C) to produce mutations. Miller recommends, for example, treating for 5 to 60 minutes with 30 µl EMS per 2 ml of cell suspension in 0.1 M Tris buffer at pH 7.5 at a temperature of 37°C. These mutagenesis conditions may be modified in an obvious manner. The selection of AHV-resistant mutants takes place on minimal agar which typically contains 2 to 10 mM AHV. The corresponding alleles may then be cloned and subjected to a sequence determination and the protein variants coded by these alleles subjected to an activity determination. Optionally, the mutants produced may also be used directly. The word "directly" means that the mutants produced can be used for the production of L-threonine in a process according to the invention or that further modifications to increase the performance characteristics of these mutants, such as for example attenuating threonine-degradation or overexpression of the threonine operon, may be performed.

In the same way, the methods of *in vitro* mutagenesis may also be used, as described, for example, in the well-known manual by Sambrook et al. (Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 1989). Corresponding methods are also commercially available in the form of so-called "kits" such as, for example, the "QuikChange Site-Directed Mutagenesis Kit" supplied by Stratagene (La Jolla, USA) and described by Papworth et al. (Strategies 9(3), 3-4 (1996)).

These mutagenesis methods may naturally also be applied to other genes, alleles or strains or problems and tasks such

as, for example, the production and isolation of mutants which are resistant to L-threonine.

Preferred thrA alleles are those which code for aspartate kinase I - homoserine dehydrogenase I variants which have at least 40%, at least 45%, at least 50%, at least 55% or at least 60% of the homoserine dehydrogenase activity in the presence of 10 mM of L-threonine and/or which have at least 70%, at least 75% or at least 80% of the homoserine dehydrogenase activity in the presence of 1 mM of L-threonine, in comparison to the activity in the absence of L-threonine. Optionally, the aspartate kinase activity of the aspartate kinase I - homoserine dehydrogenase I variants in the presence of 10 mM of L-threonine is at least 60%, at least 65%, at least 70%, at least 75% or at least 80% of the activity in the absence of L-threonine.

In addition, bacteria from the family Enterobacteriaceae which contain a stop codon chosen from the group opal, ochre and amber, preferably amber, in the rpoS gene and a t-RNA suppressor chosen from the group opal suppressor, ochre suppressor and amber suppressor, preferably amber suppressor, are suitable. The amber mutation is preferably at position 33 corresponding to the amino acid sequence of the RpoS gene product. supE is preferably used as amber suppressor. These bacteria are described in PCT/EP02/02055. A strain which contains the described mutation in the rpoS gene and the suppressor supE is obtainable, under accession number DSM 15189, from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

The nucleotide sequence of the rpoS gene can be found in the prior art. The nucleotide sequence of the rpoS gene corresponding to accession number AE000358 is given as SEQ ID NO. 1. The amino acid sequence of the associated RpoS gene product or protein is given in SEQ ID NO. 2. The nucleotide sequence of a rpoS allele which contains a stop codon of the amber type at the site in the nucleotide sequence

corresponding to position 33 of the amino acid sequence of the RpoS gene product or protein, corresponding to SEQ ID NO. 1 or SEQ ID NO. 2, is reproduced in SEQ ID NO. 3. The suppressor supE is described in the prior art and is given as
5 SEQ ID NO. 4.

In addition, suitable bacteria from the family Enterobacteriaceae are those which are not able to degrade threonine under aerobic culture conditions nor to use it as a source of nitrogen. Aerobic culture conditions are understood
10 to be those in which the oxygen partial pressure in the fermentation culture is greater than (>) 0%, for 90%, preferably 95%, very particularly preferably 99% of the fermentation time. A strain of this type is, for example, the strain KY10935 described by Okamoto (Bioscience,
15 Biotechnology and Biochemistry 61(11), 1877-1882 (1997)). Strains which are not able to degrade threonine with the elimination of nitrogen generally have an attenuated threonine dehydrogenase (EC 1.1.1.103) coded by the tdh gene. The enzyme was described by Aronson et al. (The Journal of
20 Biological Chemistry 264(9), 5226-5232 (1989)). Attenuated tdh genes are described, for example, in Ravnikar and Somerville (Journal of Bacteriology, 1986, 168(1), 434-436) in US 5, 705,371, in WO 02/26993 and in Komatsubara (Bioprocess Technology 19, 467-484 (1994)).
25 A suitable tdh allele is described in US 5,538,873 and is obtainable, in the form of strain B-3996 under accession number 1876, from the Russian National Collection of Industrial Microorganisms (VKPM, Moscow, Russia). Another tdh allele is described in US 5,939,307 and is obtainable in the
30 form of strain kat-13 under accession number NRRL B-21593, from the Agricultural Research Service Patent Culture Collection (Peoria, Illinois, USA). Finally, a tdh allele is described in WO 02/26993 and is deposited at NRRL in the form of strain TH21.97, under accession number NRRL B-30318. The
35 allele tdh-1::cat1212 coding for a defective threonine dehydrogenase is obtainable from the E. coli Genetic Stock

Centre (New Haven, Conn., USA) under accession number CGSC 6945.

In addition, bacteria from the family Enterobacteriaceae which possess an at least partial isoleucine requirement 5 ("leaky" phenotype) which can be compensated for by the addition of L-isoleucine at a concentration of at least 10, 20 or 50 mg/l or L-threonine at a concentration of at least 50, 100 or 500 mg/l, are also suitable.

A requirement or auxotrophy is generally understood to mean 10 that a strain has completely lost, for example, an enzyme activity, due to a mutation of a wild type function and requires the addition of a supplement, for example an amino acid, in order to grow. Partial requirement or partial auxotrophy is referred to when, for example, the activity of 15 an enzyme from the biosynthetic pathway for an amino acid is impaired or attenuated but not completely switched off, due to a mutation of a wild type function. Strains with partial requirement typically have, in the absence of the supplement, a reduced, i.e. greater than (>) 0% and less than (<) 90%, 20 50%, 25% or 10%, rate of growth as compared to that of the wild type. In the literature, this connection is also called a "leaky" phenotype or "leakiness" (Griffiths et al.: An Introduction to Genetic Analysis, 6th edition, 1996, Freeman and Company, New York, USA).

25 A strain with this type of partial isoleucine requirement is described, for example, in WO 01/14525 and is deposited at KCCM in the form of strain DSM9906, under accession number KCCM 10168. Threonine-releasing or -producing strains with an isoleucine requirement generally have an attenuated threonine 30 deaminase coded by the *ilvA* gene (E.C. number 4.3.1.19). Threonine deaminase is also known by the name threonine dehydratase. An attenuated *ilvA* gene which causes partial isoleucine auxotrophy is described, for example, in US 4,278,765 and is obtainable from VKPM in the form of strain 35 MG442, deposited under accession number B-1682.

Another attenuated ilvA gene is described, for example in WO 00/09660 and is obtainable from KCCM in the form of strain DSM 9807, deposited under accession number KCCM-10132. Further attenuated ilvA genes are described in Komatsubara 5 (Bioprocess Technology 19, 467-484 (1994)).

The amino acid sequence of a suitable and new threonine deaminase comprises, for example, the sequence in SEQ ID NO. 6, wherein any amino acid except glutamic acid may be present at position 286. Glutamic acid is preferably replaced by 10 lysine (E286K).

The expression "amino acid" is intended to mean in particular the proteinogenic L-amino acids, including the salts thereof, chosen from the group L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophane, L-proline and L-arginine.

SEQ ID NO. 8 gives the amino acid sequence of a threonine deaminase which contains the amino acid lysine at position 20 286; the associated nucleotide sequence is given as SEQ ID NO. 7. This contains the nucleobase adenine at position 856.

A different suitable threonine deaminase is the variant described by Lee et al. (Journal of Bacteriology 185 (18), 5442-5451 (2003)), in which serine at position 97 is replaced 25 by phenylalanine (S97F). Further suitable threonine deaminases are the variants described by Fischer and Eisenstein (Journal of Bacteriology 175 (20), 6605-6613 (1993)), which possess at least one amino acid substituent chosen from the group: replacement of asparagine at position 30 46 by aspartic acid (N46D), replacement of alanine at position 66 by valine (A66V), replacement of proline at position 156 by serine (P156S), replacement of glycine at position 248 by cysteine (G248C) and replacement of aspartic acid at position 266 by tyrosine (D266Y).

By using insertion or deletion mutagenesis of at least one base pair or nucleotide or by insertion or deletion of at least one codon in the coding region or by incorporating a stop codon by transition or transversion mutagenesis in the 5 coding region of the *ilvA* gene, alleles in which expression of the *ilvA* gene is generally completely switched off can be isolated. This method can also be transferred to other genes, alleles or open reading frames such as, for example, the *tdh* gene coding for threonine dehydrogenase.

- 10 In addition, suitable bacteria from the family Enterobacteriaceae are those which are resistant to inhibition by L-threonine and/or L-homoserine during growth. Threonine-resistant strains and the preparation thereof are described, for example, in Astaurova et al. (*Prikladnaya* 15 *Biokhimia Microbiologiya* (1985), 21(5), 485 as English translation: *Applied Biochemistry and Microbiology* (1986), 21, 485-490)). The mutant described by Astaurova is resistant to 40 mg/ml of L-threonine. Furthermore, the strain 472T23, which can grow in the presence of 5 mg/ml of L- 20 threonine and is at the same time resistant to L-homoserine, is described, for example, in US 5,175,107. Strain 472T232 is obtainable from VKPM under accession number BKIIM B-2307 and from ATCC under the number ATCC 9801. Furthermore, WO 00/09660 describes strain DSM 9807 which can grow on a solid 25 nutrient medium which contains 7% of L-threonine. Strain DSM 9807 is obtainable from KCCM under accession number KCCM-10132. Finally, WO 01/14525 describes strain DSM 9906 which can grow in a medium which contains 60% to 70% of a L-threonine fermentation mother liquor. Strain DSM 9906 is 30 obtainable from KCCM under accession number KCCM-10168.

It is known (see EP 0994 190 A2 and Livshits et al. (*Research in Microbiology* 154, 123-135 (2003))), that resistance to L-threonine and L-homoserine is brought about by enhancing the *rhtA* gene. Enhancement can be produced by increasing the copy 35 number of the gene or by using the *rhtA23* mutation.

EP 0 994 190 A2 discloses that enhancement of the rhtB gene causes resistance to L-homoserine and L-threonine, in particular to L-homoserine, and improves threonine production. The minimum inhibition concentration of 250 µg/ml can be raised to 30000 µg/ml by overexpressing the RhtB gene product in a strain called N99.

EP 1 013 765 A1 discloses that enhancement of the rhtC gene brings about resistance to L-threonine and improves threonine production. A strain which is designated resistant to L-threonine is one which can grow in the presence of a concentration of at least 30 mg/ml of L-threonine on a minimal agar. Furthermore, it is disclosed that enhancement of the rhtB gene brings about resistance to L-homoserine and improves threonine production. A strain which is designated as resistant to L-homoserine is one which can grow in the presence of a concentration of at least 5 mg/ml of L-homoserine on a minimal agar. Strains are described in the patent application mentioned which are resistant to 10 mg/ml of L-homoserine and resistant to 50 mg/ml of L-threonine. US 4,996,147 describes the strain H-4581 which is resistant to 15 g/l of homoserine. Strain H-4581 is obtainable from the National Institute of Advanced Industrial Science and Technology, under accession number FERM BP-1411.

EP 1 016 710 A2 discloses that enhancing the open reading frame or gene yfiK or yeaS brings about resistance to L-threonine and L-homoserine. The minimum inhibition concentration with respect to L-homoserine of 500 µg/ml can be increased to 1000 µg/ml and with respect to L-threonine can be increased from 30000 µg/ml to 40000 µg/ml by overexpressing the YfiK gene product in a strain called TG1. The minimum inhibition concentration with respect to L-homoserine of 500 µg/ml can be increased to 1000 µg/ml and with respect to L-threonine can be increased from 30000 µg/ml to 50000 µg/ml by overexpressing the YeaS gene product. Furthermore, it is shown, in the patent application

mentioned, that threonine production can be improved by overexpressing the YfiK gene product.

In accordance with these technical instructions, strains were prepared which can grow in the presence of \geq (at least) ≥ 5 5 g/l, ≥ 10 g/l, ≥ 20 g/l, ≥ 30 g/l, ≥ 40 g/l, ≥ 50 g/l, ≥ 60 g/l and ≥ 70 g/l of L-threonine, i.e. are resistant to L-threonine and are suitable for the production of L-threonine in a process according to the invention.

Strains which have at least the following features are 10 particularly suitable for use in the process according to the invention:

- a) a threonine-insensitive aspartate kinase I - homoserine dehydrogenase I, which is optionally present overexpressed, and
- 15 b) a stop codon chosen from the group opal, ochre and amber, preferably amber in the rpoS gene, and a t-RNA suppressor chosen from the group opal suppressor, ochre suppressor and amber suppressor, preferably amber suppressor.
- 20 In addition, strains which have at least the following features are particularly suitable for use in the process according to the invention:
 - a) a threonine-insensitive aspartate kinase I - homoserine dehydrogenase I, which is optionally present overexpressed,
 - 25 b) are not able, under aerobic culture conditions, to degrade threonine, preferably due to the attenuation of threonine dehydrogenase,
 - c) an at least partial isoleucine requirement, and
 - 30 d) can grow in the presence of at least 5 g/l of threonine.

Strains which have at least the following features are very particularly suitable for use in the process according to the invention:

- a) a threonine-insensitive aspartate kinase I - homoserine dehydrogenase I, which is optionally present overexpressed,
- b) a stop codon chosen from the group opal, ochre and amber, preferably amber in the *rpoS* gene, and a t-RNA suppressor chosen from the group opal suppressor, ochre suppressor and amber suppressor,
- c) are not able, under aerobic culture conditions, to degrade threonine, preferably due to the attenuation of threonine dehydrogenase,
- d) an at least partial isoleucine requirement, and
- e) can grow in the presence of at least 5 g/l of threonine.

In addition, bacteria used for the process according to the invention may also have one or more of the following features:

- attenuation of phosphoenolpyruvate-carboxykinase (PEP-carboxykinase) coded by the *pckA* gene as is described for example in WO 02/29080,
- attenuation of phosphoglucose isomerase coded by the *pgi* gene (Froman et al. Molecular and General Genetics 217(1):126-31 (1989)).
- attenuation of the *YtfP* gene product coded by open reading frame *ytfP* as is described for example in WO 02/29080,
- attenuation of the *YjfA* gene product coded by open reading frame *yjfA* as is described for example in WO 02/29080,
- attenuation of pyruvate oxidase coded by the *poxB* gene, as is described for example in WO 02/36797,

- attenuation of the YjgF gene product coded by open reading frame yjgF as is described for example in PCT/EP03/14271. The yjgF Orf from Escherichia coli has been described by Wasinger VC. and Humphery-Smith I. (FEMS Microbiology Letters 169(2): 375-382 (1998)), Volz K. (Protein Science 8(11): 2428-2437 (1999)) and Parsons et al. (Biochemistry 42(1): 80-89 (2003)). The associated nucleotide and amino acid sequences are available in public data banks under accession number AE000495. For the sake of better clarity, these are given as SEQ ID NO. 9 and SEQ ID NO. 10.
- enhancement of transhydrogenase coded by the genes pntA and pntB as is described for example in EP 0 733 712 A1,
- enhancement of phosphoenolpyruvate synthase coded by the pps gene as is described for example in EP 0 877 090 A1,
- enhancement of phosphoenolpyruvate carboxylase coded by the ppc gene as is described for example in EP 0 723 011 A1, and
- enhancement of regulator RseB coded by the rseB gene as is described for example in EP 1382685. The regulator RseB has been described by Missiakas et al. (Molecular Microbiology 24(2), 355-371 (1997)), De Las Penas et al. (Molecular Microbiology 24(2): 373-385 (1997)) and Collinet et al. (Journal of Biological Chemistry 275(43): 33898-33904 (2000)). The associated nucleotide and amino acid sequences are available from public data banks under accession number AE000343.
- enhancement of galactose-proton symporters (= galactose permease) coded by the galP gene as is described for example in DE 10314618.0. The galP gene and its function have been described by Macpherson et al. (The Journal of Biological Chemistry 258(7): 4390-4396 (1983)) and Venter et al. (The Biochemical Journal 363(Pt 2): 243-252 (2002)). The associated nucleotide and amino acid sequences are

available from public data banks under accession number AE000377.

- The ability to make use of saccharose as a source of carbon. Genetic determinants for the utilization of saccharose are described in the prior art, for example in FR-A-2559781, in Debabov (In: Proceedings of the IV International Symposium on Genetics of Industrial Microorganisms 1982. Kodansha Ltd, Tokyo, Japan, p 254-258), Smith and Parsell (Journal of General Microbiology 87, 129-140 (1975)) and Livshits et al. (In: Conference on Metabolic Bacterial Plasmids. Tartusk University Press, Tallin, Estonia (1982), p 132-134 and 144-146) and in US 5,705,371. The genetic determinants for saccharose utilization of strain H155 described by Smith and Parsell were transferred by conjugation into a nalidixic acid-resistant mutant of Escherichia coli K-12 and the corresponding transconjugants deposited as DSM 16293 on the 16th March 2004 at the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Genetic determinants for saccharose utilization are also present in the strain 472T23 described in US 5,631,157 and this is obtainable from ATCC under the name ATCC 9801. Another genetic determinant for saccharose utilization was described by Bockmann et al. (Molecular and General Genetics 235, 22-32 (1992)) and is disclosed under the name csc system.
- enhancement of the YedA gene product coded by open reading frame yedA as is described for example in WO 03/044191.
- growth in the presence of at least 0.1 to 0.5 mM or at least 0.5 to 1 mM of borrelidin (borrelidin resistance) as is described in US 5,939,307. Strain kat-13 which is resistant to borrelidin is obtainable from NRRL under accession number NRRL B-21593.

- growth in the presence of at least 2 to 2.5 g/l or at least 2.5 to 3 g/l of diaminosuccinic acid (diaminosuccinic acid resistance) as described in WO 00/09661. The strain DSM 9806 which is resistant to diaminosuccinic acid is
5 obtainable from KCCM under accession number KCCM-10133.
- growth in the presence of at least 30 to 40 mM or at least 40 to 50 mM of α -methylserine (α -methylserine resistance) as described in WO 00/09661. Strain DSM 9806 which is resistant to α -methylserine is obtainable from KCCM under
10 accession number KCCM-10133.
- growth in the presence of at most 30 mM or at most 40 mM or at most 50 mM of fluoropyruvic acid (fluoropyruvic acid sensitivity) as described in WO 00/09661. The strain DSM 9806 which is sensitive to fluoropyruvic acid is obtainable
15 from KCCM under accession number KCCM-10133.
- growth in the presence of at least 210 mM or at least 240 mM or at least 270 mM or at least 300 mM of L-glutamic acid (glutamic acid resistance) as described in WO 00/09660. Strain DSM 9807 which is resistant to glutamic acid is
20 obtainable from KCCM under accession number KCCM-10132.
- an at least partial requirement for methionine. A strain with an at least partial methionine requirement is the strain H-4257 described in US 5,017,483 and is obtainable from the National Institute of Advanced Industrial Science and Technology under accession number FERM BP-984. The requirement can be compensated by adding at least 25, 50 or
25 100 mg/l of L-methionine.
- an at least partial requirement for m-diaminopimelic acid. A strain with an at least partial m-diaminopimelic acid requirement is the strain H-4257 described in US 5,017,483 and this is obtainable from the National Institute of Advanced Industrial Science and Technology under accession
30 number FERM BP-984. The requirement can be compensated by

adding at least 25, 50 or 100 mg/l of m-diaminopimelic acid.

- growth in the presence of at least 100 mg/l of rifampicin (rifampicin resistance) as described in US 4,996,147. The strain H-4581 which is resistant to rifampicin is obtainable from the National Institute of Advanced Industrial Science and Technology under accession number FERM BP-1411.
5
- growth in the presence of at least 15 g/l of L-lysine (lysine resistance) as described in US 4,996,147. The strain H-4581 which is resistant to L-lysine is obtainable from the National Institute of Advanced Industrial Science and Technology under accession number FERM BP-1411.
10
- growth in the presence of at least 15 g/l of methionine (methionine resistance) as described in US 4,996,147. The strain H-4581 which is resistant to methionine is obtainable from the National Institute of Advanced Industrial Science and Technology under accession number FERM BP-1411.
15
- growth in the presence of at least 15 g/l of L-aspartic acid (aspartic acid resistance) as described in US 4,996,147. The strain H-4581 which is resistant to L-aspartic acid is obtainable from the National Institute of Advanced Industrial Science and Technology under accession number FERM BP-1411.
20
- enhancement of pyruvate carboxylase coded by the pyc gene. Suitable pyc genes or alleles are, for example, those from Corynebacterium glutamicum (WO 99/18228, WO 00/39305 and WO 02/31158), Rhizobium etli (US 6,455,284), Bacillus subtilis
25 (EP 1092776). Optionally, the pyc gene from other microorganisms which contain an endogenous pyruvate carboxylase may also be used, such as for example Methanobacterium thermoautotrophicum or Pseudomonas fluorescens.
30

When using saccharose-containing nutrient media, the strains are provided with the genetic determinants for saccharose utilization.

The expression "enhancement" in this connection describes the
5 increase in intracellular activity or concentration of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA by, for example, increasing the copy number of the open reading frame, gene or allele or open reading frames, genes or alleles by at least one (1) copy, by
10 using a strong promoter or a gene or allele which codes for a corresponding enzyme or protein with high activity and optionally by combining these steps.

When using the measure of enhancement and also when using the measure of attenuation, the use of endogenous genes, alleles
15 or open reading frames is generally preferred. "Endogenous genes" or "endogenous nucleotide sequences" are understood to be the genes or open reading frames or alleles and nucleotide sequences present in the population of a species.

When using plasmids to increase the copy number, these are
20 optionally stabilized by one or more genetic loci chosen from the group comprising the *parB* locus of the plasmid R1 described by Rasmussen et al. (Molecular and General Genetics 209 (1), 122-128 (1987)), Gerdes et al. (Molecular Microbiology 4 (11), 1807-1818 (1990)) and Thistedt und
25 Gerdes (Journal of Molecular Biology 223 (1), 41-54 (1992)), the *flm* locus of the F plasmid described by Loh et al. (Gene 66 (2), 259-268 (1988)), the *par* locus of the plasmid pSC101 described by Miller et al. (Gene 24 (2-3), 309-315 (1983)), the *cer* locus of the plasmid ColE1 described by Leung et al.
30 (DNA 4 (5), 351-355 (1985)), the *par* locus of the plasmid RK2 described by Sobecky et al. (Journal of Bacteriology 178 (7), 2086-2093 (1996)) and Roberts and Helinsky (Journal of Bacteriology 174 (24), 8119-8132 (1992)), the *par* locus of the plasmid RP4 described by Eberl et al. (Molecular
35 Microbiology 12 (1), 131-141 (1994)) and the *parA* locus of

the plasmid R1 described by Gerdes and Molin (Journal of Molecular Biology 190 (3), 269- 279 (1986)), Dam and Gerdes (Journal of Molecular Biology 236 (5), 1289- 1298 (1994)) and Jensen et al. (Proceedings of the National Academy of Sciences USA 95 (15), 8550-8555 (1998)).

As a result of enhancement, in particular overexpression, the activity or concentration of the corresponding protein or enzyme is generally increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, at most 1000% or 2000%, 10 with respect to that of the wild type protein or to the activity or concentration of the protein in the starting microorganism.

To produce an enhancement, expression of the genes or the catalytic or functional properties of the enzymes or proteins 15 are increased, for example. Optionally, the two measures may be combined.

Thus, for example, the copy number of the corresponding genes can be increased by at least one (1), or the promoter and regulation region or the ribosome binding site which is 20 located upstream of the structure gene can be mutated. Expression cassettes which are incorporated upstream of the structure gene act in the same way. In addition it is possible to increase expression during the course of fermentative L-threonine production by the use of inducible 25 promoters. Expression is also improved by measures to extend the lifetime of the m-RNA. Furthermore, the enzyme activity can also be enhanced by inhibiting degradation of the enzyme protein. The genes or gene constructs may either be present in the plasmids with different copy numbers or integrated and 30 amplified in the chromosome. Alternatively, moreover, overexpression of the relevant genes can be achieved by modifying the composition of the medium and culture management.

The expression "attenuation" in this connection describes the reduction in or switching off of the intracellular activity or concentration of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or an open reading frame or gene or allele which codes for a corresponding enzyme with a lower activity or inactivates the corresponding enzyme or protein or gene and optionally by combining these measures.

As a result of attenuation, the activity or concentration of the corresponding protein or enzyme is generally lowered to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10%, 0 to 5% or 0 to 1% or 0 to 0.1% of the activity or concentration of the wild type protein or of the activity or concentration of the protein in the starting microorganism.

To produce an attenuation, for example, expression of the genes or open reading frames or the catalytic or functional properties of the enzymes or proteins are lowered or switched off. Optionally, the two measures may be combined.

Gene expression can be reduced by suitable culture management, by genetic modification (mutation) of the signal structures of gene expression or also by antisense-RNA techniques. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. Information about this can be found by a person skilled in the art, inter alia, for example in Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)), in Carrier and Keasling (Biotechnology Progress 15: 58-64 (1999)), Franch and Gerdes (Current Opinion in Microbiology 3: 159-164 (2000)) and in well-known textbooks on genetics and molecular biology, for example the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or the book by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations which lead to a modification, for example a reduction, in the catalytic properties of enzyme proteins are disclosed in the prior art. The following may be mentioned as examples: the papers by Qiu and Goodman (Journal of

- 5 Biological Chemistry 272: 8611-8617 (1997)), Yano et al. (Proceedings of the National Academy of Sciences of the United States of America 95: 5511-5515 (1998)), Wente and Schachmann (Journal of Biological Chemistry 266: 20833-20839 (1991)). Summaries and reviews may be found in well-known
10 textbooks on genetics and molecular biology such as e.g. the book by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Suitable mutations are transitions, transversions, insertions and deletions of at least one (1) base pair or nucleotide.

- 15 Depending on the effect of the amino acid exchange caused by the mutation on the enzyme activity, reference is made to missense mutations or nonsense mutations. Missense mutations lead to the replacement of a given amino acid in a protein for another, wherein the amino acid replacement is in
20 particular non-conservative. This impairs the functionality or activity of the protein and reduces it to a value of 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10%, 0 to 5%, 0 to 1% or 0 to 0.1%. A nonsense mutation leads to a stop codon in the coding region of the gene and thus to the premature termination of
25 translation. Insertions or deletions of at least one base pair in a gene lead to frame shift mutations which then means that the wrong amino acids are incorporated or translation is prematurely terminated. As a result of the mutation, a stop codon is produced in the coding region and this also leads to
30 premature termination of translation. Deletion of at least one (1) or more codons also leads typically to the complete failure of enzyme activity or function.

- Strains which are suitable for the process according to the invention are, inter alia, strain BKIIM B-3996 described in
35 US 5,175,107, strain KCCM-10132 described in WO 00/09660 and isoleucine-requiring mutants of the strain kat-13 described

in WO 98/04715. Optionally, strains with the features mentioned, can be adapted for use in the process according to the invention, in particular by incorporating a stop codon in the *rpoS* gene, for example an amber codon at the site
5 corresponding to position 33 in the amino acid sequence for the RpoS protein and simultaneously incorporating a corresponding t-RNA suppressor, for example supE.

Strains which are suitable for the process according to the invention can also be identified by determining the
10 nucleotide sequence of the *rpoS* gene in a L-threonine-eliminating strain of *Escherichia coli*. For this purpose, the *rpoS* gene is cloned or amplified with the aid of the polymerase chain reaction (PCR) and the nucleotide sequence is determined. If the *rpoS* gene contains a stop codon then,
15 in a second step, it is checked whether it also contains a corresponding t-RNA suppressor. Optionally, the strain with the properties described above and identified in this way is provided with one or more of the other properties specified such as overexpression of the *thrA* allele, attenuation of
20 threonine degradation taking place under aerobic conditions, introduction of a mutation in the *ilvA* gene causing an at least partial isoleucine requirement or growth in the presence of at least 5 g/l of threonine.

The properties and features mentioned can be transferred into
25 the desired strain by transformation, transduction or conjugation.

In the method of transformation, isolated genetic material, typically DNA, is introduced into a target strain. In the case of bacteria of the family Enterobacteriaceae such as
30 e.g. *Escherichia coli* the DNA for this purpose is incorporated in plasmid-DNA or phage-DNA and this is then transferred into the target strain. The corresponding methods and working instructions are adequately well-known from the prior art and are described in detail, for example, in the
35 manual by J. Sambrook (*Molecular Cloning, A Laboratory*

Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989).

Defined mutations can be transferred into suitable strains with the aid of the method of gene or allele replacement 5 using conditional replicating plasmids. In a defined mutation at least the position in the chromosome, preferably the exact position of the modification of the nucleobase(s) and the type of modification (replacement, i.e. transition or transversion, insertion or deletion) is known. Optionally, 10 the corresponding DNA is first sequenced, using the normal methods. A normal method for producing a gene or allele replacement is described by Hamilton et al. (Journal of Bacteriology 171: 4617-4622 (1989)), in which the temperature-sensitive replicating pSC101 derivative pMAK705 15 is used. Alleles from the plasmid can be transferred to the chromosome using this method. Chromosomal alleles are transferred to the plasmid in the same way. Other methods described in the prior art, such as for example the method described by Martinez-Morales et al. (Journal of Bacteriology 20 181: 7143-7148 (1999)), the method described by Boyd et al. (Journal of Bacteriology 182: 842-847 (2000)) or the method described in WO 01/77345, may also be used.

This method can be used, inter alia to introduce rpoS alleles which contain for example stop codons, suppressor genes such 25 as for example supE, attenuated tdh alleles which contain for example deletions, attenuated ilvA alleles, thrA alleles which code for "feed back" resistant aspartate kinase I - homoserine dehydrogenase I variants, the rhtA23 mutation, attenuated pck alleles, attenuated alleles of the ytfP ORFs, 30 attenuated yjfA ORFs, attenuated poxB alleles, attenuated yjgF ORFs into the desired strains.

In the method of transduction, a genetic feature from a donor strain is transferred to a target strain using a bacteriophage. This method is part of the prior art and is 35 described for example in textbooks such as the book by E. A.

Birge (Bacterial and Bacteriophage Genetics, 4th ed., Springer Verlag, New York, USA, 2000).

In the case of *Escherichia coli* the bacteriophage P1 is typically used for generalized transduction (Lennox, Virology 5 1, 190-206 (1955)). A review of methods of generalised transduction is given in the article "Generalised Transduction" by M. Masters, which is contained within the text book by F. C. Neidhard (*Escherichia coli and Salmonella Cellular and Molecular Biology*, 2nd ed., ASM Press, 10 Washington, DC, USA, 1996). Practical instructions are given in the manual by J. H. Miller (*A Short Course In Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, Cold Spring Harbor Laboratory Press, New York, USA, 1992) or the manual by P. Gerhardt 15 "Manual of Methods for General Bacteriology" (American Society for Microbiology, Washington, DC, USA, 1981).

Using the method of transduction, resistance-promoting or other dominant genetic properties such as for example antibiotics resistance (for example kanamycin resistance, 20 chloramphenicol resistance, rifampicin resistance or borrelidin resistance), resistance to antimetabolites (for example α -amino- β -hydroxyvaleric acid-resistance, α -methyl-serine-resistance or diaminosuccinic acid-resistance), resistance to metabolites (for example threonine resistance, 25 homoserine resistance, glutamic acid resistance, methionine resistance, lysine resistance or aspartic acid resistance) or also the ability to utilize saccharose can be transferred into suitable target strains.

The method of transduction is also suitable for introducing 30 non-selectable genetic properties such as, for example, amino acid auxotrophies or requirements (for example an isoleucine requirement, methionine requirement or m-diamino pimelic acid requirement), vitamin requirements or sensitivities to antimetabolites (for example sensitivity to fluoropyruvic acid) 35 into target strains. For this purpose, *E. coli* strains

are used which contain the transposon Tn10 or Tn10kan on the chromosome, at spacings of approximately one minute. These strains are known under the expression "Singer Collection" or "Singer/Gross Collection" (Singer et al., Microbiological Reviews 53, 1-24, 1989). These strains are generally available from the E. coli Genetic Stock Center at Yale University (New Haven, CT, USA). Further information can be found in the article by M. K. B. Berlyn et al. "Linkage Map of Escherichia coli K-12, Edition 9", which is contained within the textbook by F. C. Neidhard (Escherichia coli and Salmonella Cellular and Molecular Biology, 2nd ed., ASM Press, Washington, DC, USA, 1996). In a similar way, genetic properties which are not selectable (for example fluoropyruvic acid sensitivity, suppressor mutations) and also those where the mutation site is not known, can be transferred into a variety of strains. Instructions for this process can be found, inter alia, in the textbook by J. Scaife et al. (Genetics of Bacteria, Academic Press, London, UK, 1985), in the article mentioned above by M. Masters and in the manual mentioned above by J. H. Miller. The tetracyclin resistance gene introduced with the transposon Tn10 may optionally be removed again using the method described by Bochner et al. (Journal of Bacteriology 143, 926-933 (1980)).

In the method of conjugation, genetic material is transferred from a donor to a target by cell-cell contact. Conjugative transfer of the F-factor (F: fertility), conjugative gene transfer using Hfr strains (Hfr: high frequency of recombination) and strains which contain a F'-factor (F': F prime), are among the classical processes of genetics. Reviews can be found, inter alia, in the standard work by F. C. Neidhard (Escherichia coli and Salmonella Cellular and Molecular Biology, 2nd ed., ASM Press, Washington, DC, USA, 1996). Practical instructions are given for example, in the manual by J. H. Miller (A Short Course In Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and

Related Bacteria, Cold Spring Harbor Laboratory Press, New York, USA, 1992) or the manual by P. Gerhardt "Manual of Methods for General Bacteriology" (American Society for Microbiology, Washington, DC, USA, 1981). F-, F' and Hfr strains are generally available from the E. coli Genetic Stock Center at Yale University (New Haven, CT, USA).

The method of conjugation was used, for example, to transfer the mutation thrC1010 described by Thèze and Saint-Girons (Journal of Bacteriology 118, 990-998 (1974)) into the strain MG442 (Debabov, Advances in Biochemical Engineering/Biotechnology 79, 113-136 (2003)). In the prior art, for example in Schmid et al. (Journal of Bacteriology 151, 68-76 (1982)) or Smith and Parsell (Journal of General Microbiology 87, 129-140 (1975)) and Livshits et al. (In: Conference on Metabolic Bacterial Plasmids. Tartusk University Press, Tallin, Estonia (1982), p 132-134 and 144-146,) conjugative plasmids are described which carry the ability to utilize saccharose. Thus, Debabov (In: Proceedings of the IVth International Symposium on Genetics of Industrial Microorganisms 1982. Kodansha Ltd, Tokyo, Japan, p 254-258) reports on the design of threonine-producing strains in which the ability to utilize saccharose was incorporated by using conjugation.

What is claimed is:

1. A process for the preparation of L-threonine using L-threonine producing bacteria from the family Enterobacteriaceae, wherein
 - 5 a) the bacterium is inoculated into at least a first nutrient medium and cultivated,
 - b) some of the fermentation broth is abstracted, wherein more than 90 vol.% of the total volume of the fermentation broth remains in the fermentation container, then
 - 10 c) the remaining fermentation broth is topped up with one or more further nutrient media, wherein the further nutrient medium or further nutrient media contains at least one source of carbon, at least one source of nitrogen and at least one source of phosphorus, and cultivation is continued under conditions which enable the formation of L-threonine,
 - d) steps b) and c) are optionally performed several times, and
 - 20 e) the concentration of the source(s) of carbon is adjusted to a maximum of 30 g/l during cultivation in accordance with step c) and/or d).
2. A process according to claim 1, wherein cultivation step
 - 25 (a) is performed by the batch process.
3. A process according to claim 1, wherein cultivation step (a) is performed by the fed batch process, wherein at least one added nutrient medium is used.
4. A process according to claim 1, 2 or 3, wherein less than 8 vol.% of the fermentation broth is abstracted.
 - 30

5. A process according to claim 1, 2 or 3, wherein less than 5 vol.% of the fermentation broth is abstracted.
6. A process according to claim 1, 2 or 3, wherein less than 2 vol.% of the fermentation broth is abstracted.
- 5 7. A process according to claim 1, 2 or 3, wherein the L-threonine formed is purified.
8. A process according to claim 1, wherein the source of carbon is one or more compounds chosen from the group saccharose, molasses from sugar beet or sugar cane, 10 fructose, glucose, starch hydrolysate, cellulose hydrolysate, arabinose, maltose, xylose, acetic acid, ethanol and methanol.
9. A process according to claim 1, wherein the source of nitrogen is one or more organic nitrogen containing 15 substances or substance mixtures chosen from the group peptones, yeast extract, meat extract, malt extract, corn steep liquor, soy bean flour and urea and/or one or more inorganic compounds chosen from the group ammonia, ammonium containing salts and salts of nitric acid.
- 20 10. A process according to claim 9, wherein the ammonium containing salts and salts of nitric acid are ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate, ammonium nitrate, potassium nitrate and potassium sodium nitrate.
- 25 11. A process according to claim 1, wherein the source of phosphorus is phosphoric acid or the alkali metal or alkaline earth metal salts or polymers thereof or phytic acid.
12. A process according to claim 11, wherein the alkali 30 metal salts of phosphoric acid are potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium containing salts.

13. A process according to claim 1, wherein the bacteria from the family Enterobacteriaceae are the species Escherichia coli.
14. A process according to claim 1, wherein the bacterium from the family Enterobacteriaceae contain at least one thrA gene or allele which codes for a threonine insensitive, aspartate kinase I - homoserine dehydrogenase I.
15. A process according to claim 1, wherein the bacterium from the family Enterobacteriaceae contains a stop codon chosen from the group opal, ochre and amber, preferably amber, in the rpoS gene and a t-RNA suppressor chosen from the group opal suppressor, ochre suppressor and amber suppressor, preferably amber suppressor.
16. A process according to claim 1, wherein steps b) and c) are repeated, in accordance with d), 0 to 100 times, preferably 2 to 80 times, preferably 4 to 50 times and particularly preferably 5 to 30 times.
17. A process according to claim 1, wherein the time between complete abstraction of the fermentation broth down to more than 90 vol.% of the total volume and complete topping up to about 100% with nutrient media is at most 5 hours.
18. A process according to claim 17, wherein complete topping up with nutrient media takes at most 2 hours.
19. A process according to claim 1, wherein, in the fed nutrient medium or fed nutrient media, the phosphorus to carbon ratio (P/C ratio) is adjusted to at most 4; at most 3; at most 2; at most 1.5; at most 1; at most 0.7; at most 0.5; at most 0.48; at most 0.46; at most 0.44; at most 0.42; at most 0.40; at most 0.38; at most 0.36; at most 0.34; at most 0.32; at most 0.30.

20. A process according to claim 1, wherein the withdrawn culture broth is provided with oxygen or an oxygen containing gas until the concentration of the source of carbon drops to less than 2 g/l; less than 1 g/l; less than 0.5 g/l.
- 5
21. A process according to claim 19, wherein the L threonine formed is purified.
22. A process according to claim 19, wherein at least 90% of the biomass is first removed from the culture withdrawn in step (b) and then at least 90% of the water is removed.
- 10
23. A process according to claim 1, 2 or 3, wherein the concentration of the source of carbon during the culture process is adjusted to at most 20, 10 or 5 g/l.
- 15 24. A process according to claim 1, 2 or 3, wherein the concentration of the source of carbon during the culture process is adjusted to at most 5 or 2 g/l.
25. A process according to claim 1, 2 or 3, wherein the concentration of the source of carbon during the culture process is adjusted to at most 5 g/l.
- 20
26. A process according to claim 1, 2 or 3, wherein the concentration of the source of carbon during the culture process is adjusted to at most 2 g/l.
27. A process according to claim 1, 2 or 3, wherein the yield of L threonine formed, with respect to the source of carbon used, is at least 31 wt. %.
- 25
28. A process according to claim 1, 2 or 3, wherein the yield of L threonine formed, with respect to the source of carbon used, is at least 37%.

29. A process according to claim 1, 2 or 3, wherein the yield of L threonine formed, with respect to the source of carbon used, is at least 42%.
30. A process according to claim 1, 2 or 3, wherein the yield of L threonine formed, with respect to the source of carbon used, is at least 48 wt. %.
- 5
31. A process according to claim 1, 2 or 3, wherein L threonine is formed with a space time yield of 5.0 to more than 8.0 g/l per hr.
- 10 32. A process according to claim 1, 2 or 3, wherein L threonine is formed with a space time yield of 3.5 to more than 5.0 g/l per hr.
33. A process according to claim 1, 2 or 3, wherein L threonine is formed with a space time yield of 2.5 to 15 more than 3.5 g/l per hr.
- 15
34. A process according to claim 1, wherein a fed batch process is used in cultivation step a), and that L threonine is formed with a space time yield of at least 2.5 to 5.0 g/l per hr.
- 20 35. Saccharose utilizing transeconjugants of Escherichia coli K 12 deposited as DSM 16293 at the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).
36. A process according to claim 1, 2 or 3, wherein strains are used which have at least the following features:
- 25
- a) a threonine insensitive aspartate kinase I homoserine dehydrogenase I, which is optionally present overexpressed, and
- b) a stop codon chosen from the group opal, ochre and amber, preferably amber in the rpos gene and a t
- 30

~~RNA suppressor chosen from the opal suppressor, ochre suppressor and amber suppressor.~~

37. A process according to claim 1, 2 or 3, wherein strains are used which have at least the following features:

- 5 a) a threonine insensitive aspartate kinase I homoserine dehydrogenase I, which is optionally present overexpressed,
- b) are not able to degrade threonine under aerobic conditions,
- 10 c) an at least partial isoleucine requirement, and
- d) growth in the presence of at least 5 g/l of threonine.

38. A process according to claim 1, 2 or 3, wherein strains are used which have at least the following features:

- 15 a) a threonine insensitive aspartate kinase I homoserine dehydrogenase I, which is optionally present overexpressed,
- b) a stop codon chosen from the group opal, ochre and amber, preferably amber in the rpoS gene and a t
- 20 RNA suppressor chosen from the group opal suppressor, ochre suppressor and amber suppressor,
- c) are not able to degrade threonine under aerobic conditions, preferably due to attenuation of threonine dehydrogenase,
- 25 d) an at least partial isoleucine requirement, and
- e) growth in the presence of at least 5 g/l of threonine.

39. A process according to claim 36, 37 or 38, wherein the strain used also contains one or more features chosen from the group
- 39.1 attenuation of phosphoenol pyruvate carboxykinase coded by the ~~pckA~~ gene,
- 5 39.2 attenuation of phosphoglucose isomerase coded by the ~~pgi~~ gene,
- 39.3 attenuation of the YtfP gene product coded by open reading frame ~~ytfP~~,
- 10 39.4 attenuation of the Yjfa gene product coded by open reading frame ~~yjfa~~,
- 39.5 attenuation of pyruvate oxidase coded by the ~~poxB~~ gene,
- 15 39.6 attenuation of the YjgF gene product coded by open reading frame ~~yjgF~~,
- 39.7 enhancement of transhydrogenase coded by the genes ~~pntA~~ and ~~pntB~~,
- 39.8 enhancement of phosphoenolpyruvate synthase coded by the ~~pps~~ gene,
- 20 39.9 enhancement of phosphoenolpyruvate carboxylase coded by the ~~ppe~~ gene,
- 39.10 enhancement of regulator RseB coded by the ~~rseB~~ gene,
- 25 39.11 enhancement of galactose proton symporters coded by the ~~galP~~ gene,
- 39.12 the ability to use saccharose as a source of carbon,

- 39.13 enhancement of the YedA gene product coded by open reading frame yedA,
- 39.14 growth in the presence of at least 0.1 to 0.5 mM or at least 0.5 bis 1 mM of borrelidin (borrelidin resistance),
5
- 39.15 growth in the presence of at least 2 to 2.5 g/l or at least 2.5 to 3 g/l of diaminosuccinic acid (diaminosuccinic acid resistance),
- 39.16 growth in the presence of at least 30 to 40 mM or
10 at least 40 to 50 mM of α -methylserine (α -methylserine resistance),
- 39.17 growth in the presence of at most 30 mM or at most 40 mM or at most 50 mM of fluoropyruvic acid (fluoropyruvic acid sensitivity),
- 15 39.18 growth in the presence of at least 210 mM or at least 240 mM or at least 270 mM or at least 300 mM of L Glutamic acid (glutamic acid resistance),
- 39.19 an at least partial requirement for methionine,
20 39.20 an at least partial requirement for m diamino pimelic acid,
- 39.21 growth in the presence of at least 100 mg/l of rifampicin (rifampicin resistance),
- 39.22 growth in the presence of at least 15 g/l of L lysine (lysine resistance),
- 25 39.23 growth in the presence of at least 15 g/l methionine (methionine resistance),
- 39.24 growth in the presence of at least 15 g/l of L aspartic acid (aspartic acid resistance), and

39.25 ~~enhancement of pyruvate carboxylase coded by the pyc gene.~~

40. (New) A process for the preparation of L-threonine using bacteria of the Enterobacteriaceae family, comprising:

- 5 f) inoculating and culturing bacteria of the Enterobacteriaceae family in at least a first nutrient medium, said culturing taking place in a fermentation container under conditions allowing for the formation of L-threonine;
- 10 g) abstracting some of the fermentation broth from the culture prepared in step a), wherein more than 90 vol% of the total volume of the fermentation broth remains in said fermentation container;
- 15 h) topping up the fermentation broth remaining in the fermentation container after the abstraction of step b) with at least one additional nutrient medium, wherein said additional nutrient medium contains at least one source of carbon, at least one source of nitrogen and at least one source of phosphorus, and wherein the concentration of carbon in said fermentation broth is adjusted to a maximum of 30 g/l; and
- 20 d) after the topping up of step c), continuing to culture said bacteria under conditions which allow for the formation of L-threonine.

25 41. (New) The process of claim 40, wherein said culturing in step a) is carried out by a batch process.

30 42. (New) The process of claim 40, wherein said culturing in step a) is performed by a fed batch process in which nutrient medium is added to said fermentation container.

43. (New) The process of claim 40, wherein less than 2 vol% of fermentation broth is abstracted in step b).

44. (New) The process of claim 40, further comprising
5 purifying said L-threonine from said fermentation broth.

45. (New) The process of claim 40, wherein said source of carbon is one or more compounds chosen from the group consisting of: saccharose, molasses from sugar beet or
10 sugar cane, fructose, glucose, starch hydrolysate, cellulose hydrolysate, arabinose, maltose, xylose, acetic acid, ethanol and methanol.

46. (New) The process of claim 40, wherein said source of
15 nitrogen comprises:

- a) one or more organic nitrogen-containing substances or substance mixtures selected from the group consisting of: peptones; yeast extract; meat extract; malt extract; corn steep liquor; soy bean flour; and urea; and/or
- b) one or more inorganic compounds chosen from the group consisting of: ammonia; ammonium-containing salts; and salts of nitric acid.

25 47. (New) The process of claim 40, wherein said source of phosphorus is selected from the group consisting of: phosphoric acid; an alkali metal or alkaline earth metal salt or polymer of phosphoric acid; and phytic acid.

30 48. (New) The process of claim 40, wherein said bacteria of the Enterobacteriaceae family are of the species Escherichia coli.

49. (New) The process of claim 40, wherein steps b) and c)
are repeated 5-30 times.

50. (New) The process of claim 40, wherein complete topping
up with nutrient media takes at most 2 hours.

51. (New) The process of claim 40, wherein said nutrient
feed medium has a phosphorus to carbon ratio (P/C ratio)
selected from: not more than 4; not more than 3; not
10 more than 2; not more than 1.5; not more than 1; not
more than 0.7; not more than 0.5; not more than 0.48;
not more than 0.46; not more than 0.44; not more than
0.42; not more than 0.40; not more than 0.38; not more
than 0.36; not more than 0.34; not more than 0.32; and
15 not more than 0.30.

52. (New) The process of claim 40, wherein the culture broth
removed is provided with oxygen or an oxygen-containing
gas until the concentration of the source of carbon
20 falls below a value selected from: 2 g/l; 1 g/l; and 0.5
g/l.

53. (New) The process of claim 52, further comprising
purifying said L-threonine from said fermentation broth.

25

54. (New) The process of claim 53, further comprising:

a) removing at least 90% of the biomass from the
culture withdrawn in step (b); and

b) then removing at least 90% of the remaining water.

30

55. (New) The process of claim 40, wherein the concentration
of the source of carbon during the culture is adjusted
to a value selected from: not more than 20; not more
than 10; not more than 5 g/l and not more than 2 g/l.

56. (New) The process of claim 40, wherein the yield of L-threonine formed, based on the source of carbon employed, is selected from a value of: at least 31%; at least 37%; at least 42%; and at least 48%.

5 57. (New) The process of claim 40, wherein L-threonine is formed with a space/time yield having a value selected from: 1.5 to 2.5 g/l per h; 2.5 to 3.5 g/l per h; 3.5 to 10 5.0 g/l per h; and more than 8.0 g/l per h.

58. (New) The process of claim 40, wherein said bacteria of the Enterobacteriaceae family comprise one or more of the following features:

- 15 a) a threonine-insensitive aspartate kinase I - homoserine dehydrogenase I; and
- b) an rpos gene with a stop codon selected from the group consisting of: opal; ochre; and amber; and a t-RNA suppressor selected from the group consisting of: the opal suppressor; the ochre suppressor; and the amber suppressor.

20 59. (New) The process of claim 58, wherein said bacteria of the Enterobacteriaceae family further comprise one or 25 more of the following features:

- e) an incapability, under aerobic culture conditions, of breaking down threonine,
- f) at least a partial need for isoleucine, and
- g) a capacity to grow in the presence of at least 5 30 g/l threonine.

60. (New) The process of claim 58, wherein said bacteria of the Enterobacteriaceae family further comprise one or more of the following features:

- a) attenuation of phosphoenol pyruvate carboxykinase, which is coded for by the pckA gene;
- b) attenuation of phosphoglucose isomerase, which is coded for by the pgi gene;
- c) attenuation of the YtfP gene product, which is coded for by the open reading frame ytfP;
- d) attenuation of the YjfA gene product, which is coded for by the open reading frame yjfA;
- e) attenuation of pyruvate oxidase, which is coded for by the poxB gene;
- f) attenuation of the YjgF gene product, which is coded for by the open reading frame yjgF;
- g) enhancement of transhydrogenase, which is coded for by the genes pntA and pntB;
- h) enhancement of phosphoenol pyruvate synthase, which is coded for by the pps gene;
- i) enhancement of phosphoenol pyruvate carboxylase, which is coded for by the ppc gene;
- j) enhancement of the regulator RseB, which is coded for by the rseB gene;
- k) enhancement of the galactose proton symporter, which is coded for by the galP gene;
- l) an ability to use sucrose as a source of carbon;
- m) enhancement of the YedA gene product, which is coded for by the open reading frame yedA;

- n) growth in the presence of at least 0.1 to 0.5 mM or at least 0.5 to 1 mM borrelidin (borrelidin resistance);
- 5 o) growth in the presence of at least 2 to 2.5 g/l or at least 2.5 to 3 g/l diaminosuccinic acid (diaminosuccinic acid resistance);
- p) growth in the presence of at least 30 to 40 mM or at least 40 to 50 mM α -methylserine (α -methylserine resistance);
- 10 q) growth in the presence of not more than 30 mM or not more than 40 mM or not more than 50 mM fluoropyruvic acid (fluoropyruvic acid sensitivity);
- r) growth in the presence of at least 210 mM or at least 240 mM or at least 270 mM or at least 300 mM L-glutamic acid (glutamic acid resistance);
- 15 s) at least a partial need for methionine;
- t) at least a partial need for m-diaminopimelic acid;
- 20 u) growth in the presence of at least 100 mg/l rifampicin (rifampicin resistance);
- v) growth in the presence of at least 15 g/l L-lysine (lysine resistance);
- 25 w) growth in the presence of at least 15 g/l methionine (methionine resistance);
- x) growth in the presence of at least 15 g/l L-aspartic acid (aspartic acid resistance); or
- y) enhancement of pyruvate carboxylase, which is coded for by the pyc gene.

Abstract

The invention provides an improved process for the fermentative preparation of L-threonine using L-threonine-producing bacteria from the family Enterobacteriaceae.

IAP5 Rec'd PCT/PTO 10 FEB 2006

1

10/567749

SEQUENCE LISTING:

5 <110> Degussa AG
 <120> Process for the preparation of L-threonine
 10 <130> 030235 BT
 <160> 10
 <170> PatentIn version 3.1
 15 <210> 1
 <211> 993
 <212> DNA
 <213> Escherichia coli
 20 <220>
 <221> CDS
 <222> (1)..(990)
 <223> rpos gene
 25 <400> 1
 atg agt cag aat acg ctg aaa gtt cat gat tta aat gaa gat gcg gaa 48
 Met Ser Gln Asn Thr Leu Lys Val His Asp Leu Asn Glu Asp Ala Glu
 1 5 10 15
 30 ttt gat gag aac gga gtt gag gtt ttt gac gaa aag gcc tta gta gaa 96
 Phe Asp Glu Asn Gly Val Glu Val Phe Asp Glu Lys Ala Leu Val Glu
 20 25 30
 cag gaa ccc agt gat aac gat ttg gcc gaa gag gaa ctg tta tcg cag 144
 35 Gln Glu Pro Ser Asp Asn Asp Leu Ala Glu Glu Leu Leu Ser Gln
 35 40 45
 gga gcc aca cag cgt ttg gac gcg act cag ctt tac ctt ggt gag 192
 40 Gly Ala Thr Gln Arg Val Leu Asp Ala Thr Gln Leu Tyr Leu Gly Glu
 50 55 60
 att ggt tat tca cca ctg tta acg gcc gaa gaa gtt tat ttt gcg 240
 Ile Gly Tyr Ser Pro Leu Leu Thr Ala Glu Glu Val Tyr Phe Ala
 65 70 75 80
 45 cgt cgc gca ctg cgt gga gat gtc gcc tct cgc cgc cggt atg atc gag 288
 Arg Arg Ala Leu Arg Gly Asp Val Ala Ser Arg Arg Arg Met Ile Glu
 85 90 95
 50 agt aac ttg cgt ctg gtg gta aaa att gcc cgc cgt tat ggc aat cgt 336
 Ser Asn Leu Arg Leu Val Val Lys Ile Ala Arg Arg Tyr Gly Asn Arg
 100 105 110
 55 ggc ctg gcg ttg ctg gac ctt atc gaa gag ggc aac ctg ggg ctg atc 384
 Gly Leu Ala Leu Leu Asp Leu Ile Glu Glu Gly Asn Leu Gly Leu Ile
 115 120 125
 cgc gcg gta gag aag ttt gac ccg gaa cgt ggt ttc cgc ttc tca aca 432
 60 Arg Ala Val Glu Lys Phe Asp Pro Glu Arg Gly Phe Arg Phe Ser Thr
 130 135 140
 tac gca acc tgg tgg att cgc cag acg att gaa cgg gcg att atg aac 480
 Tyr Ala Thr Trp Trp Ile Arg Gln Thr Ile Glu Arg Ala Ile Met Asn
 145 150 155 160
 65

	caa acc cgt act att cgt ttg ccg att cac atc gta aag gag ctg aac		528
	Gln Thr Arg Thr Ile Arg Leu Pro Ile His Ile Val Lys Glu Leu Asn		
	165	170	175
5	gtt tac ctg cga acc gca cgt gag ttg tcc cat aag ctg gac cat gaa		576
	Val Tyr Leu Arg Thr Ala Arg Glu Leu Ser His Lys Leu Asp His Glu		
	180	185	190
10	cca agt gcg gaa gag atc gca gag caa ctg gat aag cca gtt gat gac		624
	Pro Ser Ala Glu Glu Ile Ala Glu Gln Leu Asp Lys Pro Val Asp Asp		
	195	200	205
15	gtc agc cgt atg ctt cgt ctt aac gag cgc att acc tcg gta gac acc		672
	Val Ser Arg Met Leu Arg Leu Asn Glu Arg Ile Thr Ser Val Asp Thr		
	210	215	220
20	ccg ctg ggt ggt gat tcc gaa aaa gcg ttg ctg gac atc ctg gcc gat		720
	Pro Leu Gly Gly Asp Ser Glu Lys Ala Leu Leu Asp Ile Leu Ala Asp		
	225	230	235
	240		
25	gaa aaa gag aac ggt ccg gaa gat acc acg caa gat gac gat atg aag		768
	Glu Lys Glu Asn Gly Pro Glu Asp Thr Thr Gln Asp Asp Asp Met Lys		
	245	250	255
30	cag agc atc gtc aaa tgg ctg ttc gag ctg aac gcc aaa cag cgt gaa		816
	Gln Ser Ile Val Lys Trp Leu Phe Glu Leu Asn Ala Lys Gln Arg Glu		
	260	265	270
35	gtg ctg gca cgt cga ttc ggt ttg ctg ggg tac gaa gcg gca aca ctg		864
	Val Leu Ala Arg Arg Phe Gly Leu Leu Gly Tyr Glu Ala Ala Thr Leu		
	275	280	285
40	gaa gat gta ggt cgt gaa att ggc ctc acc cgt gaa cgt gtt cgc cag		912
	Glu Asp Val Gly Arg Glu Ile Gly Leu Thr Arg Glu Arg Val Arg Gln		
	290	295	300
45	att cag gtt gaa ggc ctg cgc cgt ttg cgc gaa atc ctg caa acg cag		960
	Ile Gln Val Glu Gly Leu Arg Arg Leu Arg Glu Ile Leu Gln Thr Gln		
	305	310	315
	320		
50	ggg ctg aat atc gaa gcg ctg ttc cgc gag taa		993
	Gly Leu Asn Ile Glu Ala Leu Phe Arg Glu		
	325	330	
55	<210> 2		
	<211> 330		
	<212> PRT		
	<213> Escherichia coli		
60	<400> 2		
	Met Ser Gln Asn Thr Leu Lys Val His Asp Leu Asn Glu Asp Ala Glu		
	1	5	10
	15		
65	Phe Asp Glu Asn Gly Val Glu Val Phe Asp Glu Lys Ala Leu Val Glu		
	20	25	30
	35	40	45
70	Gln Glu Pro Ser Asp Asn Asp Leu Ala Glu Glu Leu Leu Ser Gln		
	50	55	60
	65	70	75
	80		

Arg Arg Ala Leu Arg Gly Asp Val Ala Ser Arg Arg Arg Met Ile Glu
 85 90 95

5 Ser Asn Leu Arg Leu Val Val Lys Ile Ala Arg Arg Tyr Gly Asn Arg
 100 105 110

Gly Leu Ala Leu Leu Asp Leu Ile Glu Glu Gly Asn Leu Gly Leu Ile
 115 120 125

10 Arg Ala Val Glu Lys Phe Asp Pro Glu Arg Gly Phe Arg Phe Ser Thr
 130 135 140

Tyr Ala Thr Trp Trp Ile Arg Gln Thr Ile Glu Arg Ala Ile Met Asn
 145 150 155 160

15 Gln Thr Arg Thr Ile Arg Leu Pro Ile His Ile Val Lys Glu Leu Asn
 165 170 175

20 Val Tyr Leu Arg Thr Ala Arg Glu Leu Ser His Lys Leu Asp His Glu
 180 185 190

Pro Ser Ala Glu Glu Ile Ala Glu Gln Leu Asp Lys Pro Val Asp Asp
 195 200 205

25 Val Ser Arg Met Leu Arg Leu Asn Glu Arg Ile Thr Ser Val Asp Thr
 210 215 220

Pro Leu Gly Gly Asp Ser Glu Lys Ala Leu Leu Asp Ile Leu Ala Asp
 225 230 235 240

30 Glu Lys Glu Asn Gly Pro Glu Asp Thr Thr Gln Asp Asp Asp Met Lys
 245 250 255

35 Gln Ser Ile Val Lys Trp Leu Phe Glu Leu Asn Ala Lys Gln Arg Glu
 260 265 270

Val Leu Ala Arg Arg Phe Gly Leu Leu Gly Tyr Glu Ala Ala Thr Leu
 275 280 285

40 Glu Asp Val Gly Arg Glu Ile Gly Leu Thr Arg Glu Arg Val Arg Gln
 290 295 300

Ile Gln Val Glu Gly Leu Arg Arg Leu Arg Glu Ile Leu Gln Thr Gln
 305 310 315 320

45 Gly Leu Asn Ile Glu Ala Leu Phe Arg Glu
 325 330

50 <210> 3
 <211> 993
 <212> DNA
 <213> Escherichia coli

55 <220>
 <221> Allele
 <222> (1)..(990)
 <223> rpoS allele

60 <220>
 <221> misc_feature
 <222> (97)..(99)
 <223> amber codon

65 <400> 3
 atgagtcaga atacgctgaa agttcatgat ttaaatgaag atgcggatt tgatgagaac

	ggagttgagg ttttgacga aaaggccta gtagaatagg aaccagtga taacgatttgcg	120
5	gccgaagagg aactgttatac gcagggagcc acacagcgtg tggtggacgc gactcagcttac	180
	taccttggtg agattggta ttcaccactg ttaacggccg aagaagaagt ttatggcg	240
	cgtcgacac tgcgtggaga tgctgcctct cgccgcccga tgatcgagag taacttgcgt	300
10	ctgggtgtaa aaattgccccg ccgttatggc aatcggtgtc tggcggtgct ggaccttatac	360
	gaagagggca acctggggct gatccgcgcg gtagagaagt ttgacccgga acgtggtttc	420
15	cgcttctcaa catacgcaac ctgggtggatt cgccagacga ttgaacgggc gattatgaac	480
	caaaccgtta ctattcgaaa gccgattcac atcgtaaagg agctgaacgt ttacctgcga	540
	accgcacgtg agttgtccca taagctggac catgaaccaa gtgcggaaaga gatcgacag	600
20	caactggata agccagttga tgacgtcagc cgtatgcctc gtcttaacga ggcattacc	660
	tcggtagaca ccccgctggg tggtgattcc gaaaaagcgt tgctggacat cctggccgat	720
25	gaaaaagaga acggtccgga agataccacg caagatgacg atatgaagca gagcatcgca	780
	aatggctgt tcgagctgaa cgccaaacag cgtgaagtgc tggcacgtcg attcggtttg	840
	ctgggttacg aagcggcaac actggaagat gtaggtcgtg aaattggcct cacccgtgaa	900
30	cgtgttcgcc agattcaggt tgaaggcctg cgccgtttgc gcgaaatcct gcaaacgcag	960
	gggctgaata tcgaagcgct gttccgcgag taa	993
	<210> 4	
35	<211> 75	
	<212> DNA	
	<213> Escherichia coli	
	<220>	
40	<221> tRNA	
	<222> (1)..(75)	
	<223> supE allele	
	<400> 4	
45	tgggtatcg ccaagcggtt aggcaccggaa ttcttaattcc ggcattccga ggttcgaatc	60
	ctcgtaaaaa agcca	75

<210>	5		
<211>	1545		
<212>	DNA		
<213>	Escherichia coli		
5			
<220>			
<221>	CDS		
<222>	(1)..(1542)		
10	<223> ilvA-Gen		
<400>	5		
atg gct gac tcg caa ccc ctg tcc ggt gct ccg gaa ggt gcc gaa tat			48
Met Ala Asp Ser Gln Pro Leu Ser Gly Ala Pro Glu Gly Ala Glu Tyr			
15 1 5 10 15			
tta aga gca gtg ctg cgc gcg ccg gtt tac gag gcg gcg cag gtt acg			96
Leu Arg Ala Val Leu Arg Ala Pro Val Tyr Glu Ala Ala Gln Val Thr			
20 20 25 30			
ccg cta caa aaa atg gaa aaa ctg tcg tcg cgt ctt gat aac gtc att			144
Pro Leu Gln Lys Met Glu Lys Leu Ser Ser Arg Leu Asp Asn Val Ile			
35 35 40 45			
ctg gtg aag cgc gaa gat cgc cag cca gtg cac agc ttt aag ctg cgc			192
Leu Val Lys Arg Glu Asp Arg Gln Pro Val His Ser Phe Lys Leu Arg			
50 50 55 60			
ggc gca tac gcc atg atg gcg ggc ctg acg gaa gaa cag aaa gcg cac			240
Gly Ala Tyr Ala Met Ala Gly Leu Thr Glu Glu Gln Lys Ala His			
65 65 70 75 80			
ggc gtg atc act gct tct gcg ggt aac cac gcg cag ggc gtc gcg ttt			288
Gly Val Ile Thr Ala Ser Ala Gly Asn His Ala Gln Gly Val Ala Phe			
85 85 90 95			
tct tct gcg cgg tta ggc gtg aag gcc ctg atc gtt atg cca acc gcc			336
Ser Ser Ala Arg Leu Gly Val Lys Ala Leu Ile Val Met Pro Thr Ala			
100 100 105 110			
acc gcc gac atc aaa gtc gac gcg gtg cgc ggc ttc ggc ggc gaa gtg			384
Thr Ala Asp Ile Lys Val Asp Ala Val Arg Gly Phe Gly Gly Glu Val			
115 115 120 125			
ctg ctc cac ggc gcg aac ttt gat gaa gcg aaa gcc aaa gcg atc gaa			432
Leu Leu His Gly Ala Asn Phe Asp Glu Ala Lys Ala Lys Ala Ile Glu			
130 130 135 140			
ctg tca cag cag ggg ttc acc tgg gtg ccg ccg ttc gac cat ccg			480
Leu Ser Gln Gln Gln Gly Phe Thr Trp Val Pro Pro Phe Asp His Pro			
145 145 150 155 160			
atg gtg att gcc ggg caa ggc acg ctg gcg ctg gaa ctg ctc cag cag			528
Met Val Ile Ala Gly Gln Gly Thr Leu Ala Leu Glu Leu Leu Gln Gln			
165 165 170 175			
gac gcc cat ctc gac cgc gta ttt gtg cca gtc ggc ggc ggc ggt ctg			576
Asp Ala His Leu Asp Arg Val Phe Val Pro Val Gly Gly Gly Leu			
180 180 185 190			
gct gct ggc gtg gcg gtg ctg atc aaa caa ctg atg ccg caa atc aaa			624
Ala Ala Gly Val Ala Val Leu Ile Lys Gln Leu Met Pro Gln Ile Lys			
195 195 200 205			

gtg atc gcc gta gaa gcg gaa gac tcc gcc tgc ctg aaa gca gcg ctg Val Ile Ala Val Glu Ala Glu Asp Ser Ala Cys Leu Lys Ala Ala Leu 210 215 220	672
5 gat gcg ggt cat ccg gtt gat ctg ccg cgc gta ggg cta ttt gct gaa Asp Ala Gly His Pro Val Asp Leu Pro Arg Val Gly Leu Phe Ala Glu 225 230 235 240	720
10 ggc gta gcg gta aaa cgc atc ggt gac gaa acc ttc cgt tta tgc cag Gly Val Ala Val Lys Arg Ile Gly Asp Glu Thr Phe Arg Leu Cys Gln 245 250 255	768
15 gag tat ctc gac gac atc atc acc gtc gat agc gat gcg atc tgt gcg Glu Tyr Leu Asp Asp Ile Ile Thr Val Asp Ser Asp Ala Ile Cys Ala 260 265 270	816
20 gcg atg aag gat tta ttc gaa gat gtg cgc gcg gtg gcg gaa ccc tct Ala Met Lys Asp Leu Phe Glu Asp Val Arg Ala Val Ala Glu Pro Ser 275 280 285	864
25 ggc gcg ctg gcg ctg gca atg aaa aaa tat atc gcc ctg cac aac Gly Ala Leu Ala Leu Ala Gly Met Lys Tyr Ile Ala Leu His Asn 290 295 300	912
30 ttc cac ggc ctg cgc tac gtc tca gaa cgc tgc gaa ctg ggc gaa cag Phe His Gly Leu Arg Tyr Val Ser Glu Arg Cys Glu Leu Gly Glu Gln 325 330 335	1008
35 cgt gaa gcg ttg ttg gcg gtg acc att ccg gaa gaa aaa ggc agc ttc Arg Glu Ala Leu Leu Ala Val Thr Ile Pro Glu Glu Lys Gly Ser Phe 340 345 350	1056
40 ctc aaa ttc tgc caa ctg ctt ggc ggg cgt tcg gtc acc gag ttc aac Leu Lys Phe Cys Gln Leu Leu Gly Gly Arg Ser Val Thr Glu Phe Asn 355 360 365	1104
45 tac cgt ttt gcc gat gcc aaa aac gcc tgc atc ttt gtc ggt gtg cgc Tyr Arg Phe Ala Asp Ala Lys Asn Ala Cys Ile Phe Val Gly Val Arg 370 375 380	1152
50 ctg agc cgc ggc ctc gaa gag cgc aaa gaa att ttg cag atg ctc aac Leu Ser Arg Gly Leu Glu Glu Arg Lys Glu Ile Leu Gln Met Leu Asn 385 390 395 400	1200
55 gac ggc ggc tac agc gtg gtt gat ctc tcc gac gac gaa atg gcg aag Asp Gly Gly Tyr Ser Val Val Asp Leu Ser Asp Asp Glu Met Ala Lys 405 410 415	1248
60 cta cac gtg cgc tat atg gtc ggc gga cgt cca tcg cat ccg ttg cag Leu His Val Arg Tyr Met Val Gly Gly Arg Pro Ser His Pro Leu Gln 420 425 430	1296
65 gaa cgc ctc tac agc ttc gaa ttc ccg gaa tca ccg ggc gcg ctg ctg Glu Arg Leu Tyr Ser Phe Glu Phe Pro Glu Ser Pro Gly Ala Leu Leu 435 440 445	1344
70 cgc ttc ctc aac acg ctg ggt acg tac tgg aac att tct ttg ttc cac Arg Phe Leu Asn Thr Leu Gly Thr Tyr Trp Asn Ile Ser Leu Phe His 450 455 460	1392

tat cgc agc cat ggc acc gac tac ggg cgc gta ctg gcg gcg ttc gaa 1440
 Tyr Arg Ser His Gly Thr Asp Tyr Gly Arg Val Leu Ala Ala Phe Glu
 465 470 475 480

5 ctt ggc gac cat gaa ccg gat ttc gaa acc ccg ctg aat gag ctg ggc 1488
 Leu Gly Asp His Glu Pro Asp Phe Glu Thr Arg Leu Asn Glu Leu Gly
 485 490 495

10 tac gat tgc cac gac gaa acc aat aac ccg gcg ttc agg ttc ttt ttg 1536
 Tyr Asp Cys His Asp Glu Thr Asn Asn Pro Ala Phe Arg Phe Phe Leu
 500 505 510

15 gcg ggt tag 1545
 Ala Gly

20 <210> 6
 <211> 514
 <212> PRT
 <213> Escherichia coli

25 <400> 6
 Met Ala Asp Ser Gln Pro Leu Ser Gly Ala Pro Glu Gly Ala Glu Tyr
 1 5 10 15

Leu Arg Ala Val Leu Arg Ala Pro Val Tyr Glu Ala Ala Gln Val Thr
 20 25 30

30 Pro Leu Gln Lys Met Glu Lys Leu Ser Ser Arg Leu Asp Asn Val Ile
 35 40 45

Leu Val Lys Arg Glu Asp Arg Gln Pro Val His Ser Phe Lys Leu Arg
 50 55 60

35 Gly Ala Tyr Ala Met Met Ala Gly Leu Thr Glu Glu Gln Lys Ala His
 65 70 75 80

40 Gly Val Ile Thr Ala Ser Ala Gly Asn His Ala Gln Gly Val Ala Phe
 85 90 95

Ser Ser Ala Arg Leu Gly Val Lys Ala Leu Ile Val Met Pro Thr Ala
 100 105 110

45 Thr Ala Asp Ile Lys Val Asp Ala Val Arg Gly Phe Gly Gly Glu Val
 115 120 125

Leu Leu His Gly Ala Asn Phe Asp Glu Ala Lys Ala Lys Ala Ile Glu
 130 135 140

50 Leu Ser Gln Gln Gln Gly Phe Thr Trp Val Pro Pro Phe Asp His Pro
 145 150 155 160

55 Met Val Ile Ala Gly Gln Gly Thr Leu Ala Leu Glu Leu Leu Gln Gln
 165 170 175

Asp Ala His Leu Asp Arg Val Phe Val Pro Val Gly Gly Gly Leu
 180 185 190

60 Ala Ala Gly Val Ala Val Leu Ile Lys Gln Leu Met Pro Gln Ile Lys
 195 200 205

Val Ile Ala Val Glu Ala Glu Asp Ser Ala Cys Leu Lys Ala Ala Leu
 210 215 220

65

Asp Ala Gly His Pro Val Asp Leu Pro Arg Val Gly Leu Phe Ala Glu
 225 230 235 240
 Gly Val Ala Val Lys Arg Ile Gly Asp Glu Thr Phe Arg Leu Cys Gln
 5 245 250 255
 Glu Tyr Leu Asp Asp Ile Ile Thr Val Asp Ser Asp Ala Ile Cys Ala
 260 265 270
 10 Ala Met Lys Asp Leu Phe Glu Asp Val Arg Ala Val Ala Glu Pro Ser
 275 280 285
 Gly Ala Leu Ala Leu Ala Gly Met Lys Lys Tyr Ile Ala Leu His Asn
 290 295 300
 15 Ile Arg Gly Glu Arg Leu Ala His Ile Leu Ser Gly Ala Asn Val Asn
 305 310 315 320
 Phe His Gly Leu Arg Tyr Val Ser Glu Arg Cys Glu Leu Gly Glu Gln
 20 325 330 335
 Arg Glu Ala Leu Leu Ala Val Thr Ile Pro Glu Glu Lys Gly Ser Phe
 340 345 350
 25 Leu Lys Phe Cys Gln Leu Leu Gly Gly Arg Ser Val Thr Glu Phe Asn
 355 360 365
 Tyr Arg Phe Ala Asp Ala Lys Asn Ala Cys Ile Phe Val Gly Val Arg
 370 375 380
 30 Leu Ser Arg Gly Leu Glu Glu Arg Lys Glu Ile Leu Gln Met Leu Asn
 385 390 395 400
 Asp Gly Gly Tyr Ser Val Val Asp Leu Ser Asp Asp Glu Met Ala Lys
 35 405 410 415
 Leu His Val Arg Tyr Met Val Gly Gly Arg Pro Ser His Pro Leu Gln
 420 425 430
 40 Glu Arg Leu Tyr Ser Phe Glu Phe Pro Glu Ser Pro Gly Ala Leu Leu
 435 440 445
 Arg Phe Leu Asn Thr Leu Gly Thr Tyr Trp Asn Ile Ser Leu Phe His
 45 450 455 460
 Tyr Arg Ser His Gly Thr Asp Tyr Gly Arg Val Leu Ala Ala Phe Glu
 465 470 475 480
 Leu Gly Asp His Glu Pro Asp Phe Glu Thr Arg Leu Asn Glu Leu Gly
 50 485 490 495
 Tyr Asp Cys His Asp Glu Thr Asn Asn Pro Ala Phe Arg Phe Phe Leu
 500 505 510
 55 Ala Gly

<210> 7
 <211> 1545
 60 <212> DNA
 <213> Escherichia coli

<220>
 <221> CDS
 <222> (1)..(1542)
 <223> ilvA-Allel
 5

<220>
 <221> mutation
 <222> (856)..(856)
 10 <223>

<400> 7 atg gct gac tcg caa ccc ctg tcc ggt gct ccg gaa ggt gcc gaa tat 15 Met Ala Asp Ser Gln Pro Leu Ser Gly Ala Pro Glu Gly Ala Glu Tyr 5 10 15	48
tta aga gca gtg ctg cgc gcg ccg gtt tac gag gcg gcg cag gtt acg 20 Leu Arg Ala Val Leu Arg Ala Pro Val Tyr Glu Ala Ala Gln Val Thr 20 25 30	96
ccg cta caa aaa atg gaa aaa ctg tcg tcg cgt ctt gat aac gtc att 25 Pro Leu Gln Lys Met Glu Lys Leu Ser Ser Arg Leu Asp Asn Val Ile 35 40 45	144
ctg gtg aag cgc gaa gat cgc cag cca gtg cac agc ttt aag ctg cgc 30 Leu Val Lys Arg Glu Asp Arg Gln Pro Val His Ser Phe Lys Leu Arg 50 55 60	192
ggc gca tac gcc atg atg gcg ggc ctg acg gaa gaa cag aaa gcg cac 35 Gly Ala Tyr Ala Met Ala Gly Leu Thr Glu Glu Gln Lys Ala His 65 70 75 80	240
ggc gtg atc act gct tct gcg ggt aac cac gcg cag ggc gtc gcg ttt 40 Gly Val Ile Thr Ala Ser Ala Gly Asn His Ala Gln Gly Val Ala Phe 85 90 95	288
tct tct gcg cgg tta ggc gtg aag gcc ctg atc gtt atg cca acc gcc 45 Ser Ser Ala Arg Leu Gly Val Lys Ala Leu Ile Val Met Pro Thr Ala 100 105 110	336
acc gcc gac atc aaa gtc gac gcg gtg cgc ggc ttc ggc ggc gaa gtg 50 Thr Ala Asp Ile Lys Val Asp Ala Val Arg Gly Phe Gly Gly Glu Val 115 120 125	384
ctg ctc cac ggc gcg aac ttt gat gaa gcg aaa gcc aaa gcg atc gaa 55 Leu Leu His Gly Ala Asn Phe Asp Glu Ala Lys Ala Lys Ala Ile Glu 130 135 140	432
ctg tca cag cag cag ggg ttc acc tgg gtg ccg ccg ttc gac cat ccg 60 Leu Ser Gln Gln Gly Phe Thr Trp Val Pro Pro Phe Asp His Pro 145 150 155 160	480
atg gtg att gcc ggg caa ggc acg ctg gcg ctg gaa ctg ctc cag cag 65 Met Val Ile Ala Gly Gln Gly Thr Leu Ala Leu Glu Leu Leu Gln Gln 165 170 175	528
gac gcc cat ctc gac cgc gta ttt gtg cca gtc ggc ggc ggc ggt ctg 70 Asp Ala His Leu Asp Arg Val Phe Val Pro Val Gly Gly Gly Leu 180 185 190	576
gct gct ggc gtg gcg gtg ctg atc aaa caa ctg atg ccg caa atc aaa 75 Ala Ala Gly Val Ala Val Leu Ile Lys Gln Leu Met Pro Gln Ile Lys 195 200 205	624

65

	gtg atc gcc gta gaa gcg gaa gac tcc gcc tgc ctg aaa gca gcg ctg Val Ile Ala Val Glu Ala Glu Asp Ser Ala Cys Leu Lys Ala Ala Leu 210 215 220	672
5	gat gcg ggt cat ccg gtt gat ctg ccg cgc gta ggg cta ttt gct gaa Asp Ala Gly His Pro Val Asp Leu Pro Arg Val Gly Leu Phe Ala Glu 225 230 235 240	720
10	ggc gta gcg gta aaa cgc atc ggt gac gaa acc ttc cgt tta tgc cag Gly Val Ala Val Lys Arg Ile Gly Asp Glu Thr Phe Arg Leu Cys Gln 245 250 255	768
15	gag tat ctc gac gac atc atc acc gtc gat agc gat gcg atc tgt gcg Glu Tyr Leu Asp Asp Ile Ile Thr Val Asp Ser Asp Ala Ile Cys Ala 260 265 270	816
20	gcg atg aag gat tta ttc gaa gat gtg cgc gcg gtg gcg aaa ccc tct Ala Met Lys Asp Leu Phe Glu Asp Val Arg Ala Val Ala Lys Pro Ser 275 280 285	864
25	ggc gcg ctg gcg ctg gga atg aaa aaa tat atc gcc ctg cac aac Gly Ala Leu Ala Leu Ala Gly Met Lys Lys Tyr Ile Ala Leu His Asn 290 295 300	912
30	ttc cac ggc ctg cgc tac gtc tca gaa cgc tgc gaa ctg ggc gaa cag Phe His Gly Leu Arg Tyr Val Ser Glu Arg Cys Glu Leu Gly Glu Gln 325 330 335	1008
35	cgt gaa gcg ttg ttg gcg gtg acc att ccg gaa gaa aaa ggc agc ttc Arg Glu Ala Leu Leu Ala Val Thr Ile Pro Glu Glu Lys Gly Ser Phe 340 345 350	1056
40	ctc aaa ttc tgc caa ctg ctt ggc ggg cgt tcg gtc acc gag ttc aac Leu Lys Phe Cys Gln Leu Leu Gly Gly Arg Ser Val Thr Glu Phe Asn 355 360 365	1104
45	tac cgt ttt gcc gat gcc aaa aac gcc tgc atc ttt gtc ggt gtg cgc Tyr Arg Phe Ala Asp Ala Lys Asn Ala Cys Ile Phe Val Gly Val Arg 370 375 380	1152
50	ctg agc cgc ggc ctc gaa gag cgc aaa gaa att ttg cag atg ctc aac Leu Ser Arg Gly Leu Glu Glu Arg Lys Glu Ile Leu Gln Met Leu Asn 385 390 395 400	1200
55	gac ggc ggc tac agc gtg gtt gat ctc tcc gac gac gaa atg gcg aag Asp Gly Gly Tyr Ser Val Val Asp Leu Ser Asp Asp Glu Met Ala Lys 405 410 415	1248
60	cta cac gtg cgc tat atg gtc ggc gga cgt cca tcg cat ccg ttg cag Leu His Val Arg Tyr Met Val Gly Gly Arg Pro Ser His Pro Leu Gln 420 425 430	1296
	gaa cgc ctc tac agc ttc gaa ttc ccg gaa tca ccg ggc gcg ctg ctg Glu Arg Leu Tyr Ser Phe Glu Phe Pro Glu Ser Pro Gly Ala Leu Leu 435 440 445	1344
	cgc ttc ctc aac acg ctg ggt acg tac tgg aac att tct ttg ttc cac Arg Phe Leu Asn Thr Leu Gly Thr Tyr Trp Asn Ile Ser Leu Phe His 450 455 460	1392

tat cgc agc cat ggc acc gac tac ggg cgc gta ctg gcg gcg ttc gaa 1440
 Tyr Arg Ser His Gly Thr Asp Tyr Gly Arg Val Leu Ala Ala Phe Glu
 465 470 475 480

5 ctt ggc gac cat gaa ccg gat ttc gaa acc ccg ctg aat gag ctg ggc 1488
 Leu Gly Asp His Glu Pro Asp Phe Glu Thr Arg Leu Asn Glu Leu Gly
 485 490 495

10 tac gat tgc cac gac gaa acc aat aac ccg gcg ttc agg ttc ttt ttg 1536
 Tyr Asp Cys His Asp Glu Thr Asn Asn Pro Ala Phe Arg Phe Phe Leu
 500 505 510

15 gcg ggt tag 1545
 Ala Gly

20 <210> 8
 <211> 514
 <212> PRT
 <213> Escherichia coli

25 <400> 8
 Met Ala Asp Ser Gln Pro Leu Ser Gly Ala Pro Glu Gly Ala Glu Tyr
 1 5 10 15

Leu Arg Ala Val Leu Arg Ala Pro Val Tyr Glu Ala Ala Gln Val Thr
 20 25 30

30 Pro Leu Gln Lys Met Glu Lys Leu Ser Ser Arg Leu Asp Asn Val Ile
 35 40 45

Leu Val Lys Arg Glu Asp Arg Gln Pro Val His Ser Phe Lys Leu Arg
 50 55 60

35 Gly Ala Tyr Ala Met Met Ala Gly Leu Thr Glu Glu Gln Lys Ala His
 65 70 75 80

40 Gly Val Ile Thr Ala Ser Ala Gly Asn His Ala Gln Gly Val Ala Phe
 85 90 95

Ser Ser Ala Arg Leu Gly Val Lys Ala Leu Ile Val Met Pro Thr Ala
 100 105 110

45 Thr Ala Asp Ile Lys Val Asp Ala Val Arg Gly Phe Gly Gly Glu Val
 115 120 125

Leu Leu His Gly Ala Asn Phe Asp Glu Ala Lys Ala Lys Ala Ile Glu
 130 135 140

50 Leu Ser Gln Gln Gln Gly Phe Thr Trp Val Pro Pro Phe Asp His Pro
 145 150 155 160

55 Met Val Ile Ala Gly Gln Gly Thr Leu Ala Leu Glu Leu Leu Gln Gln
 165 170 175

Asp Ala His Leu Asp Arg Val Phe Val Pro Val Gly Gly Gly Leu
 180 185 190

60 Ala Ala Gly Val Ala Val Leu Ile Lys Gln Leu Met Pro Gln Ile Lys
 195 200 205

65 Val Ile Ala Val Glu Ala Glu Asp Ser Ala Cys Leu Lys Ala Ala Leu
 210 215 220

Asp Ala Gly His Pro Val Asp Leu Pro Arg Val Gly Leu Phe Ala Glu
 225 230 235 240

5 Gly Val Ala Val Lys Arg Ile Gly Asp Glu Thr Phe Arg Leu Cys Gln
 245 250 255

Glu Tyr Leu Asp Asp Ile Ile Thr Val Asp Ser Asp Ala Ile Cys Ala
 10 260 265 270

Ala Met Lys Asp Leu Phe Glu Asp Val Arg Ala Val Ala Lys Pro Ser
 275 280 285

Gly Ala Leu Ala Leu Ala Gly Met Lys Lys Tyr Ile Ala Leu His Asn
 15 290 295 300

Ile Arg Gly Glu Arg Leu Ala His Ile Leu Ser Gly Ala Asn Val Asn
 305 310 315 320

20 Phe His Gly Leu Arg Tyr Val Ser Glu Arg Cys Glu Leu Gly Glu Gln
 325 330 335

Arg Glu Ala Leu Leu Ala Val Thr Ile Pro Glu Glu Lys Gly Ser Phe
 25 340 345 350

Leu Lys Phe Cys Gln Leu Leu Gly Gly Arg Ser Val Thr Glu Phe Asn
 355 360 365

Tyr Arg Phe Ala Asp Ala Lys Asn Ala Cys Ile Phe Val Gly Val Arg
 30 370 375 380

Leu Ser Arg Gly Leu Glu Glu Arg Lys Glu Ile Leu Gln Met Leu Asn
 385 390 395 400

35 Asp Gly Gly Tyr Ser Val Val Asp Leu Ser Asp Asp Glu Met Ala Lys
 405 410 415

Leu His Val Arg Tyr Met Val Gly Gly Arg Pro Ser His Pro Leu Gln
 40 420 425 430

Glu Arg Leu Tyr Ser Phe Glu Phe Pro Glu Ser Pro Gly Ala Leu Leu
 435 440 445

Arg Phe Leu Asn Thr Leu Gly Thr Tyr Trp Asn Ile Ser Leu Phe His
 45 450 455 460

Tyr Arg Ser His Gly Thr Asp Tyr Gly Arg Val Leu Ala Ala Phe Glu
 465 470 475 480

50 Leu Gly Asp His Glu Pro Asp Phe Glu Thr Arg Leu Asn Glu Leu Gly
 485 490 495

Tyr Asp Cys His Asp Glu Thr Asn Asn Pro Ala Phe Arg Phe Phe Leu
 55 500 505 510

Ala Gly

<210> 9
 60 <211> 1548
 <212> DNA
 <213> Escherichia coli

```

<220>
<221> DNA
<222> (1)..(1548)
<223>
5

<220>
<221> CDS
<222> (527)..(952)
10 <223> yjgF-Orf

<400> 9
tcgcgatctg gtactgttaag gggaaataga gatgacacac gataataaat tgcagggtga 60
15 agctattaaa cgcggcacgg taattgacca tatccccgcc cagatcggtt ttaagctgtt 120
gagtctgttc aagctgaccg aaacggatca gcgcacacc accgtctga acctgccttc 180
20 tggcgagatg ggccgcaaag atctgatcaa aatcgaaaat accttttga gtgaagatca 240
agtagatcaa ctggcattgt atgcgcgcga agccacgggtt aaccgtatcg acaactatga 300
25 agtggtgggt aaatcgcgcc caagtctgcc ggagcgcac gacaatgtgc tggctgccc 360
gaacagcaac tgtatcagcc atgccgaacc ggtttcatcc agctttgccg tgcgaaaacg 420
cgccaatgat atcgcgctca aatgcaaata ctgtaaaaa gagtttccc ataatgtgg 480
30 gctggccaat taattgcggt tggtataaaa agtctggctc cctata atg agc cag
Met Ser Gln
1

act ttt tac cgc tgt aat aaa gga gaa atc atg agc aaa act atc gcg 583
35 Thr Phe Tyr Arg Cys Asn Lys Gly Glu Ile Met Ser Lys Thr Ile Ala
5 10 15

40 acg gaa aat gca ccg gca gct atc ggt cct tac gta cag ggc gtt gat 631
Thr Glu Asn Ala Pro Ala Ile Gly Pro Tyr Val Gln Gly Val Asp
20 25 30 35

45 ctg ggc aat atg atc atc acc tcc ggt cag atc ccg gta aat ccg aaa 679
Leu Gly Asn Met Ile Ile Thr Ser Gly Gln Ile Pro Val Asn Pro Lys
40 45 50

50 acg ggc gaa gta ccg gca gac gtc gct gca cag gca cgt cag tcg ctg 727
Thr Gly Glu Val Pro Ala Asp Val Ala Ala Gln Ala Arg Gln Ser Leu
55 60 65

55 gat aac gta aaa gcg atc gtc gaa gcc gct ggc ctg aaa gtg ggc gac 775
Asp Asn Val Lys Ala Ile Val Glu Ala Ala Gly Leu Lys Val Gly Asp
70 75 80

60 atc gtt aaa act acc gtg ttt gta aaa gat ctg aac gac ttc gca acc 823
Ile Val Lys Thr Thr Val Phe Val Lys Asp Leu Asn Asp Phe Ala Thr
85 90 95

65 gta aac gcc act tac gaa gcc ttc ttc acc gaa cac aac gcc acc ttc 871
Val Asn Ala Thr Tyr Glu Ala Phe Phe Thr Glu His Asn Ala Thr Phe
100 105 110 115

70 ccg gca cgt tct tgc gtt gaa gtt gcc cgt ctg ccg aaa gac gtg aag 919
Pro Ala Arg Ser Cys Val Glu Val Ala Arg Leu Pro Lys Asp Val Lys
120 125 130

```

	att gag atc gaa gcg atc gct gtt cgt cgc taa tcttgatgga aatccgggct	972
	Ile Glu Ile Glu Ala Ile Ala Val Arg Arg	
	135 140	
5	atcatgcccg gattaagtct gatgacaaaac gcaaaatcgct ctgatgcgct acgcttatca	1032
	ggcctacgtg attcctgcaa tttattgaat ttgttggccg gataaggcat ttacgcccga	1092
10	tccggcatga acaaaaactca ctttgtctac aatctgaatc gggctatcg tgcccagtt	1152
	attcttatt gccagccgta acgacggcta tagaaccctt tcaccaactg gttaatgtc	1212
	atataccctg ccagaatcgc aaccagccac gggaaatagc ttaacggcag cgccctgtaat	1272
15	tgcagataac tggccagcgg tgaaaacggc aatgcgatcc cgacaatcat cacgatcacg	1332
	gtcatgatca ttaacggcca cgatgcacag ctctgaataa acggcacacg gcgggtgcgg	1392
20	atcatatgca caatcagcgt ttgcgacagt aagcccacca caaaccatcc cgactggaac	1452
	agcgtttgcg tttccggcgt gttggcatgg aataccacc acatcaggca aaacgtcaaa	1512
	atatcgaaga tcgagctgat cggtccgaag aagatc	1548
25	<210> 10	
	<211> 141	
	<212> PRT	
30	<213> Escherichia coli	
	<400> 10	
	Met Ser Gln Thr Phe Tyr Arg Cys Asn Lys Gly Glu Ile Met Ser Lys	
	1 5 10 15	
35	Thr Ile Ala Thr Glu Asn Ala Pro Ala Ala Ile Gly Pro Tyr Val Gln	
	20 25 30	
	Gly Val Asp Leu Gly Asn Met Ile Ile Thr Ser Gly Gln Ile Pro Val	
40	35 40 45	
	Asn Pro Lys Thr Gly Glu Val Pro Ala Asp Val Ala Ala Gln Ala Arg	
	50 55 60	
45	Gln Ser Leu Asp Asn Val Lys Ala Ile Val Glu Ala Ala Gly Leu Lys	
	65 70 75 80	
	Val Gly Asp Ile Val Lys Thr Thr Val Phe Val Lys Asp Leu Asn Asp	
	85 90 95	
50	Phe Ala Thr Val Asn Ala Thr Tyr Glu Ala Phe Phe Thr Glu His Asn	
	100 105 110	
	Ala Thr Phe Pro Ala Arg Ser Cys Val Glu Val Ala Arg Leu Pro Lys	
55	115 120 125	
	Asp Val Lys Ile Glu Ile Glu Ala Ile Ala Val Arg Arg	
	130 135 140	
60		